Investigation of the potential mechanisms behind dysfunctional labour with maternal obesity

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ABBREVIATION

%	Percentage
µ	Micro
µl	Microlitre
µm	Micrometre
µg/mL	Microgram per millilitre
15 PGDH	15-Hydroxyprostaglandin dehydrogenase
Δ	Delta
A	Alpha
ANOVA	Analysis of Variance
AA	Arachidonic acid
ALA	Alpha Linoleic acid
B	Beta
BMI	Body mass Index
BK	Big Potassium
°C	Celsius
CAP	Contractile Associated Protein
CX-43	Connexin-43
Cav-1	Caveolae 1
COX-2	Cyclooxygenase-2
CON	Control
CYP17	Cytochrome P450 17alpha-hydroxylase
CO2	Carbon dioxide
CRH	Corticotropin releasing hormone

Ca^{2+}	Calcium
cm	centimetre
Δ	delta
DHA	Decosahexaenoic acid
DPA	Decosapentaenoic acid
DAG	Diacylglycerol
D5D	Delta-5 Desaturase
EPA	Eicosapentaenoic acids
EDTA	Ethylenediaminetetra acetic acid
EP	E Prostaglandin
ECL	Electrochemiluminescence
ELISA	Enzyme Linked Immune-Sorbet Assay
eNOS	E-Nitric Oxidase Synthase
ELOVL	Elongation of very Long Chain-Fatty Acid
ε	Epsilon
FABP	Fatty Acid-Binding Protein
FFAR	Free Fatty Acid Receptor
FAME	Fatty Acid Methyl Esters
FATP	fatty acid transport protein
FFA	Free Fatty Acid
FP	F Prostaglandin
FADS1	Fatty Acid Desaturase-1 Enzyme
Gas Chromatography-FID	Gas chromatography Flame Ionised Detector
GPCRs	G-protein-coupled receptors
Γ	gamma

G	Grams
g/Kg	gram per kilogram
GC	Gas chromatography
g/s	grams per second
HSCIC	Health and Social Care Information Centre
HCL	Hydrochloride acid
HFHC	High Fat and High Cholesterol
HES	Hospital Episode Statistic
H&E	Haematoxylin and Eosin
IL	Interleukin
Ig G	Immunoglobulin G
IP-3	Inositol 1,4,5-triphosphate
Kcal/g	Calories per gram
KOH	Potassium Hydroxide
KDa	Kilodalton
K^+	Potassium
ml	millilitre
M	metre
mm	millimetre
M	Molar
MLCK	Myosin Light Chain Kinase
mRNA	messenger Ribonucleic acid
MMP	Matrix Metalloproteinase
mol/L	moles per litre
ms	millisecond

NSB	Non Specific Binding
n-3	Omega-3
n-6	Omega-6
Nf-kb	Nuclear Factor Kappa B
OA	Oleic Acid
OXTR	Oxytocin Receptor
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
PUFA	Polyunsaturated fatty acid
PPAR	Peroxisome Proliferator Activated Receptor
PCX-43	Phosphorylation Connexin43
pg/ml	pictograms per millilitre
PGFM	Prostaglandin F2 Alpha Metabolite
PGEM	Prostaglandin E2 Metabolite
PGDH	Prostaglandin dehydrogenase
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PDBu	phorbol 12,13-butyrate
PLA2	Phospholipase A2
SR	sarcoplasmic reticulum
SREBP	Sterol Regulatory Element Binding Protein
TZD	Thiazolidinediones
VEGF	Vascular Endothelial Growth Factor
ζ	Zeta

ABSTRACT

The prevalence of obesity among pregnant women in Europe varies, ranging from 8 to 25% where the UK is currently ranked top with 25% of pregnant women classified obese. Public Health Scotland identified that 50% of women booking antenatal care between January 2018 and March 2019 were overweight and obese with 30% of the pregnant women delivering their baby by caesarean section. The mechanism underpinning prolonged and dysfunctional labour with maternal obesity is currently unresolved. Maternal obesity is associated with increased lipid accumulation in the placenta and myometrium that causes uterine myocytes to be less responsive to oxytocin and $PGF_{2\alpha}$. Recently, our research group established that feeding a High-Fat, High-Cholesterol (HFHC) diet to induce obesity in rats increases omega-9 and decreases both omega-3 and omega-6 fatty acids in the plasma and liver. However, only total omega-3 PUFAs decreased significantly in the uterus which exhibited unsynchronised myometrial contractions at term labour. Decreasing omega-3 in the uterus after chronic exposure to the HFHC diet suggests that the adverse effects of obesity on myometrial contractile activity could be improved by increasing omega-3 status. Thus, a key aim of this research was to investigate further the potential mechanism(s) behind prolonged and dysfunctional labour associated with maternal obesity. To achieve this, pregnant Wistar rats were fed different diets. The first experiment was setup to try and determine whether poor contractile function of the uterus after HFHC feeding in the rat is caused by increased lipid accumulation within the myometrium. Rats were fed either a control or HFHC diet and then culled for tissue collection either at term or during parturition. The aim was to evaluate the effect of the HFHC diet on the number of vacuoles and vacuole area in the uterus as an indirect representation of lipid accumulation. Furthermore, this study also investigated the effect of different diets on the percentage of fatty acids within the plasma, liver and the uterus and to

determine whether there was any correlation between the proportion of fatty acids within the uterus (EPA, DHA, AA and OA) and prostaglandin synthesis. The quantification of plasma concentrations of $PGF_{2\alpha}$ and PGE_2 were determined by ELISA kit (Abcam), while gas chromatography-FID was used to determine the fatty acid levels in the plasma, liver and uterus. Two-way ANOVA was used to analyse the relationship between diet and time of delivery (Term or Labour) on the number of vacuole and mean area of vacuole, while one-way ANOVA was used for analysing the effect of HFHC diet on fatty acid proportion in plasma, liver and uterus. If significantly different, the Post hoc LSD statistic test was used at the $P<0.05$ level. The correlation of fatty acids within the uterus and plasma type 2 prostaglandins type were analysed by Spearman statistic test. The results of the study were that exposure to a HFHC diet significantly alters lipid accumulation in the uterus, alters plasma, liver and uterine fatty acid proportions that correlated with plasma type 2 prostaglandins. The uterus from rats fed a HFHC diet throughout pregnancy significantly increased the mean number and mean area of vacuoles compared to the control group. The HFHC group had twice the number of vacuoles compared to controls. The HFHC diet also significantly increased the proportion of saturated fatty acids and OA, but decreased the proportion of omega-3 and omega-6 in the plasma and liver, meanwhile, only omega-3 was significantly decreased in the uterus. Interestingly, changing diet from a HFHC diet to control at conception and fed throughout pregnancy increased the percentage of omega-3 in the uterus ($P< 0.05$). Since a high fat diet affects uterine fatty acid proportions, the next study aimed to investigate whether a high fat diet alters the proportion of fatty acids in the placenta as well as protein expression of PPARs and aromatase. PPARs are nuclear receptor proteins that has a function as transcription factors regulating the expression of genes associated with inflammation and lipid homeostasis. Moreover, the placenta is an important organ for the synthesis of steroid hormones including progesterone and oestrogen. As our research group established that the HFHC diet increased progesterone

production at term compared to controls it suggests maternal obesity may cause prolonged and dysfunctional labour via altered steroid synthesis. Aromatase is one of the enzymes that are essential for oestrogen synthesis, but many studies reported that it is inhibited by PPAR γ. Therefore, the hypothesis to test was that HFHC diet would affect the proportion of fatty acids within the plasma and placenta. This study also determined whether a HFHC diet increase protein expression of PPARs but decreased protein expression of aromatase in the placenta. Western blotting was used to analyse protein expression of PPARs and aromatase in the placenta, while the proportion of fatty acid in the placenta used the same method as that in the first chapter of the study. One Way ANOVA was used to analyse the different proportions of fatty acids and protein expression of PPAR between the HFHC control groups, followed by the LSD test if it significant differences were identified at the $P< 0.05\ level$. The result of this study was that exposure to the HFHC diet decreased the saturated fatty acid proportion but significantly increased DGLA compared to the control group ($P<0.05$). Moreover, rats fed the HFHC diet had significantly higher protein expression of PPAR γ compared to the lean control group ($P=0.01$).

As we had identified decreased omega-3 status in the uterus after chronic exposure to the HFHC diet it suggested that the adverse effects of obesity on myometrial contractile activity could be improved by increasing omega-3 status. Moreover, our research group established that a HFHC decreased protein expression of key contractile associated protein (CAPs) such as CX-43 and OXTR. Fatty acids, especially omega-3 PUFA's could enhance the membrane fluidity which potentially increase the coupling efficiency of G-Protein such as free fatty acid receptor (FFAR) that has the potential to modify the signalling and mRNA stability of connexin and transcription activity of the connexin gene. Furthermore, fatty acids could be taken up by fatty acid-binding protein (FABP) in coordination with CD36 receptor and transport them from membrane to the cytoplasm altering function of the CAPs in the cell. Therefore, the third

chapter of the study was to investigate the effect of different ratios of the omega-3 PUFA ALA and omega -6 PUFA LA at two different fat levels on uterine protein expression of CAPs (CX-43, Cav-1, COX-2), OXT, PCX-43) and PPARs (PPAR α, PPAR γ and PPAR δ). For this experiment pregnant rats were fed different ratios of omega-3 and omega-6 followed at two fat levels ; high omega-3 and high fat diet (1:1.5 36%), high omega-3 and low fat diet (1:1.5 18%), high omega-6 and high fat (9:1 36%), high omega-6 and low fat (9:1 18%). Western blotting was used to determine protein expression of CAPs and PPARs. Two-Way ANOVA followed by LSD determined whether there was a relationship between diet and level of fat on protein expression of CAPs and PPARs. Exposure to the high omega-3 PUFA diet significantly increased protein expression of CX-43 and Cav-1 ($P<0.05$). In contrast, the high omega-3 diet significantly increased COX-2 protein expression but only if fed at the higher 36% fat level ($P=0.02$). Statistical analysis identified an interaction between diet and the level of fat on PPAR α expression within the uterus ($P=0.02$) where the high LA high fat group (9:1 36%) had the highest expression of PPAR α in the uterus. The finding that improved omega-3 PUFA status increases uterine protein expression of CX-43, Cav-1 and COX-2 suggests that there is potential to improve myometrial contractile activity during parturition in obese pregnant women.

The OA also has the potential to alter myometrial contractions since previous results identified that the HFHC diet significantly increases OA levels in the liver and plasma. Uterine and plasma levels of $PGF_{2\alpha}$ have also been shown to have a positive correlation with OA levels in the uterus. There is evidence to suggest that OA might contribute to increased systolic calcium-free concentrations and activate Ca^{2+} dependent PKC isoenzymes to promote redistribution of protein kinase C (PKC) from the cytosol to the plasma and possibly alter the signaling pathways associated with myometrial contraction such as gap junction, prostaglandin type 2 receptor and MLCK. Thus, the fourth chapter of the study aimed to investigate the effect

of OA on myometrial contractile activity i*n* an *ex-vivo* organ bath study. Myometrial strips from term-labour pregnant rats fed a control chow diet were used in this study. Strips of myometrium were left to equilibrate for 30 to 40 minutes in an organ bath. Baseline recording of 15 minutes spontaneous uterine contractility was determined before accumulative response to increasing doses of OA, the PKC inhibitor (Go6983) or accumulation of OA in combination with either the PKC activator (PMA) or PKC inhibitor (Go6983) were recorded. The myometrial contractile activity was extracted from Lab Chart Reader software and the ordinary fit least square analysis was used to determine the correlation between OA dose and the myometrial contractile parameters and the correlation between Go6983 exposure with myometrial contractile activity. Dose-response curves were used to analyse the concentration of OA or Go6983 that inhibit 50% of myometrial contractile force or stimulate 50% of contractile (EC50) force. The Repeat Measured ANOVA statistical test was used to determine the effect of myometrial response to PMA when exposed to increasing concentrations of OA and the effect of Go6983 in combination with increasing concentrations of OA and PMA on myometrial contractile activity. The result of this experiment was that exposure to OA leads to poorly synchronized myometrial contractions and decreased frequency of contractions but increased mean and peak amplitude and time of the peak in a dose dependent manner. However, PMA in combination with increased accumulation of OA did not alter myometrial contractile activity. Meanwhile, administration of Go6983 in combination with PMA and OA had the reverse effect of myometrial contractile activity which shows increased frequency but decreased peak of amplitude ($P<0.05$). Furthermore, the individual effect of PKC inhibitor Go6983 towards uterine contractions showed a significant decrease in the peak amplitude and frequency of uterine contractions ($P<0.05$). The poorly synchronized and decreased frequency of myometrial contractions after OA administration and the reverse effect of myometrial

contraction after Go6983 administration suggests that OA might alter myometrial contraction through PKC activation.

In conclusion, besides obesity itself, fat and PUFA diets, especially LA and ALA have the ability to alter myometrial contractile activity through altered protein expression of CAPs and PPARs. Improving omega-3 status through dietary intervention is a potential treatment to prevent dysfunctional labour with maternal obesity, and acts by increasing protein expression of the CAPs such as CX-43, Cav-1 and COX-2. Furthermore, evidence suggests that OA increased the amplitude of contraction but otherwise decreased their frequency. In addition, OA induced unsynchronized myometrial contractions during parturition via PKC which might be detrimental to pregnant women.

PUBLICATION

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1. Literature Review

1.1 Maternal obesity and Its adverse outcome

Obesity, which is characterised by the excessive accumulation of body fat and recognised as a significant health concern worldwide, is indicated by a body mass index more than or equal to 30 kg/m^2 (Alison, 2016). Over a third of the global population currently suffer from being overweight or obese and if this trend continues, it is estimated that by 2030 some 38% of the world's adult population will be overweight, and 20% will be obese (Kelly et al., 2008; Stevens et al., 2012). Epidemiological studies revealed that obesity is a critical risk factor for diabetes, cardiovascular disease, cancer, and premature deaths (Haslam and James, 2005).

Data gathered by the World Health Organization (WHO, 2009) revealed the prevalence of obesity among pregnant women in Europe to vary from 8 % to 25% with the United Kingdom having the highest prevalence (Poston et al., 2016). In fact, obesity has been reported to affect one-fifth of reproductive-aged females in the United Kingdom (Alison, 2016). Health and Social Care Information Centre (HSCIC) England mentioned that more than 50% of women of reproductive aged between 25 and 44 years old are currently overweight and obese (HSCIC, 2016). A similar trend is reported by Public Health Scotland (2020) where 50% of women booked for antenatal care in Scotland hospitals between 2018 -2019 were overweight and obese with 30% of the pregnant women delivering their baby by caesarean section. Furthermore, one in five women who received antenatal care in a Glasgow maternity hospital was clinically obese (Kanagalingam et al., 2005). If the trend continues, it will have serious health related consequences and result in substantial human costs, as obesity is known to contribute to the onset of adverse pregnancy outcomes.

A number of studies have identified maternal complications in pregnancy to be associated with maternal obesity such as preeclampsia and gestational diabetes (Schrauwers and Dekker, 2009; Shaikh et al., 2010). A retrospective review of pregnant patients in South Australia with BMI>30 have a two-fold greater risk of developing preeclampsia and eight times greater risk of developing gestational diabetes compared to mothers with a normal BMI (20-24.5) (Schrauwers and Dekker, 2009). A systematic review of 13 cohort studies between 1980 to 2002 also established that an increased BMI is an independent risk factor for developing preeclampsia (O'Brien et al., 2003).

In addition, the systematic review conducted by Heslehurst et al. (2008) identified that maternal obesity exhibits a positive correlation with an increased incidence of dysfunctional labour as well as an increased number and duration of both induced labour and caesarean sections. The same study found that women with a BMI>40 require more epidurals than women who have a BMI within the normal range (Heslehurst et al., 2008). This finding is supported by the results of an observational study conducted by Kiran et al. (2005), that identified maternally obese women have a 1.5–2 times higher risk of requiring an induced labour or caesarean section. Moreover, it was observed from 60,167 deliveries in South Glamorgan between 1990 and 1999 that women with maternal obesity were at increased risk of their baby suffering shoulder dystocia, failed instrumental delivery and maternal complications (e.g. blood loss of more than 500 ml and urinary tract infections) during labour (Kiran et al., 2005). Obese mothers might require prolonged recovery periods and hospital stays due to maternal and foetal complications, which may result in increased costs when compared with mothers who experience normal deliveries (Kuhle et al., 2019). In fact, a retrospective prevalence-based study conducted in Wales from 2011–2012 found the total mean cost of health service usage throughout pregnancy and up to two months after delivery to be £3546.30 for a normal-weight

mother, while it was £4244.40 for an overweight mother and £4717.64 for an obese mother (Morgan et al., 2014). Aside from the maternal complications in pregnancy associated with obesity, maternal obesity can also lead to foetal distress, neonatal stress and stillbirth (Shaikh et al., 2010). Moreover, a systematic review and meta-analysis of studies conducted from 1966 to 2008 confirmed maternal obesity to be associated with various foetal congenital abnormalities, including poor vision, spina bifida and neural tube defects (Stothard et al., 2009). In an observational study, Jolly et al. (2003) observed 350,311 completed singleton pregnancies in the North West Thames region between 1988 and 1997 and reported another risk of maternal obesity to be the fact that obese mothers are more likely to give birth to babies with foetal macrosomia (i.e. babies with a neonatal birthweight >4 kg). The same study also reported that increased maternal glucose, triglyceride and amino acid levels can cross the haemochorial placenta, which may explain the pathogenesis of foetal macrosomia (Jolly et al., 2003).

Maternal pre-pregnancy obesity is known to be positively associated with adverse conditions in the offspring as well as with increased healthcare utilisation and short-term costs. Data gathered from 1989 to 1994 by the Canadian Institute for Health Information revealed that obese mothers have $365 higher physician costs and $1415 higher hospital costs over the first 18 years of their child's life when compared with mothers who have a normal BMI (Kuhle et al., 2019). Furthermore, maternal obesity is negatively associated with the prolactin response when the baby breastfeeds during the first week postpartum, which indicates that it might lead to the failure of early lactation (Rasmussen and Kjolhede, 2004).

Maternal obesity can represent a serious health problem due to causing pregnancy, maternal and foetal complications, which can result in increased healthcare usage and costs. One problem associated with obesity is prolonged and dysfunctional

labour, which is the leading cause of caesarean section. A Systematic review and Meta-analysis studies published in England between 2009 and 2017 shows that labour induction and caesarean section was more common among women with obesity compared than normal pregnant women (Ellis *et al*., 2019). Moreover, Retrospective cohort study in Soderhospital, Stockholm illustrated that pregnant women with BMI 30-35 group has higher number to get prostaglandin and oxytocin induction compared than normal group. Induced labour is less efficient than spontaneous labour and therefore maternal obesity who are induced had 1.5 higher risk to have caesarean section (Bjorklund et al., 2022). Thus, understanding the mechanism behind prolonged and dysfunctional labour could represent a crucial strategy for preventing complications associated with maternal obesity.

1.2 Anatomical structure of uterus and placenta

1.2.1 Uterus

The uterus is located in the female pelvis between the urinary bladder and the rectum. In an adult female, the uterus commonly measures 8 cm in length, 5 cm in width and 4 mm in thickness. The uterus is subdivided into three segments, namely the body, the cervix and the fundus. Moreover, the uterus has three tissue layers ;

a. Endometrium: The inner lining of the uterus which composed of epithelial cells, stroma cells, immune cells and endothelial cells. It consists of two regions, namely the functional endometrium and basal endometrium. The functional layers respond to reproductive hormones. Following the implantation of an embryo, the uterine endometrium is referred to as the decidua (Ochoa-Bernal and Fazleabas, 2020).

b. Myometrium: the muscle layer and is composed of smooth muscle cells. The myometrium is spontaneously active and produces regular contractions without the need of external stimuli such as hormonal or nervous input. The myometrium consists of bundles of uterine myocytes that contain three layers of smooth muscles (Verralls, 1993). Myocytes are organised into a longitudinal and circular layer in rodents, while in a human, myocytes are often described as random swirling and mixing fibres (Young, 2007). Uterine myocytes contain a sarcoplasmic reticulum, where calcium (Ca^{2+}) ions are released and contribute to an increase in myometrial contractions (Wray, 2007). Moreover, uterine myocytes express membrane-bound and cytosolic proteins that are important for the contraction and relaxation of the myometrium, including G Protein-Coupled Receptor, ion channels, and Gap junction proteins (Brenninkmeijer et al., 1999; Wray, 2007). During parturition, uterine myocytes show hyperplasia, hypertrophy, and an increase in proteins forming Gap junctions and communication between cells (Challis et al., 2011).

c. Perimetrium: the thin outer layer composed of epithelial cells (Ammer et al., 2021).

The uterus changes from pear a shape during non-pregnancy to a ball or sphere shape in the first trimester then expands to elongated cylinders in the third trimester. The growth of the uterus occurs via two processes. The first process is where oestrogen and progesterone-induces hyperplasia of the uterine smooth muscle cells within the myometrium during early pregnancy. The further process is hypertrophy of the uterine muscles later in pregnancy. The muscle enhances the content of actin, myosin, sarcoplasmic reticulum, and mitochondria which serve as the machinery used to contract the muscles during parturition (King et al., 2015).

1.2.2 Placenta

The placenta is a pregnancy organ responsible for essential functions during pregnancy such as foetal support, nourishment and protection. The rat placenta is histologically composed of the labyrinth zone, basal zone, and decidua, and material glands. In the labyrinth zone, there are three layers of trophoblasts, separating the maternal blood spaces from foetal blood vessel. The first layer is cytotrophoblasts with the microvillous surface, then the second layer is syncytiotrophoblasts located under trophectoderm. The cytotrophoblast acquires invasive capacity, invading maternal decidua and part of the myometrium. The basal zone consists of three types of differentiated cells namely spongiotrophoblasts, trophoblastic giant cell and glycogen cells. The decidua plays an essential role in the development of vascularized decidual-placental interface (Furukawa et al,, 2014).

In the past, the placenta was only known to help distribute nutrients to the foetus. However, with progress in obstetric research it has now been widely reported that the placenta represents a very metabolically active organ during parturition. The placenta can interact within and between intrauterine tissue throughout the cell to communicate cell to cell (paracrine), within the same cell (autocrine) to produce several hormones during pregnancy (endocrine) (Petraglia et al., 1996; Schuler et al., 2018). Furthermore, many studies identified that the placenta produces a large variety of molecules, including steroid hormones, such as progesterone and oestrogen, cytokines, and neuroendocrine hormones such as oxytocin and CRH (Vannuccini et al., 2016). The major source of placental hormones is located in the syncytiotrophoblast layer, which expresses the enzymes and machinery required for biosynthesis of the pregnancy related hormones. Moreover, another layer of the placenta, the trophoblast also

produces placental hormones such as chorionic gonadotropin (Kliman, 1993; Lunghi et al., 2007).

1.3. Physiology of the stage of labour

Parturition is a coordinated process of transition from quiescent myometrium to active myometrial contractions, which requires interaction between placental, foetal and maternal compartments (Vannuccini et al., 2016). Parturition is an integrated change of the uterus (myometrium, decidua, uterine dilatation) over the last trimester of pregnancy. At term labour, the uterine prostaglandins are released and increase myometrial gap junction formation and upregulate expression of the myometrial oxytocin receptors resulting in myometrial contractions. Furthermore, the interaction between inflammation, steroid hormone and paracrine molecules also contribute to the modulation of labour onset and progression (Golightly et al., 2011; Kamel, 2010).

Parturition is divided into 4 phases. The first phase is the quiescent phase. During quiescence, the uterus is kept quiescent through the activation of progesterone, prostacyclin (PGI2), and nitric oxide. All these agents mediate the increased intercellular concentration of cAMP and cGMP, which inhibit the release of intracellular calcium and cause myometrial relaxation. The second phase is the activation phase. During this phase, there is change from progesterone domination to decreased progesterone and increased oestrogen and cortisol releasing hormone (CRH), with associated increases in contractile association proteins (CAPs) including connexin-43 (CX-43) and Cyclooxygenase (COX-2), as well as up-regulation of myometrial oxytocin receptors. In this phase, the calcium-sensitive form of active PKC stimulates an increase in the concentration of intracellular Ca^{2+}. The elevated intracellular free calcium in myometrial cells increases the activity of Phospholipase A2 (PLA2), the enzyme that releases arachidonic acid from lipid membranes for the synthesis of

prostaglandins (Morrison et al., 1996). Furthermore, the intracellular Ca^{2+} ions bind with calmodulin and active Myosin Light Chain Kinase (MLCK) pathways which are vital for uterine mechanical activity (Wray, 2007). In this phase, Protein Kinase C (PKC) activation also induces phosphorylation of the actin-binding protein calponin (CaP), which allows more actin to bind myosin and enhances contraction (Ringvold and Khalil, 2017). Furthermore, another study identified that activation of PKC and tyrosine kinase are involved in the regulation of oxytocin-mediated myometrial contractions since oxytocin-stimulated contractile activity was reduced after administration of staurosporine a PKC inhibitor in an *in-vitro* isometric contraction study of human pregnant myometrium (Morrison et al., 1996).

The third phase is the stimulation phase in which uterotonics, including oxytocin, prostaglandins, and CRH, stimulate the uterus to contract and get ready for parturition. In this phase, the biochemical events in the uterus are similar to the inflammatory reaction with an increased level of cytokines (Terzidou, 2007; Vannuccini et al., 2016). Increased level of inflammatory cytokines are associated with increased protein and mRNA expression of Cyclooxygenase (COX-2) that is essential for prostaglandin type 2 production in the human amnion cells (Ackerman et al., 2005), human uterine myocytes (Rauk and Chio, 2000) and bovine placenta (Schuler et al., 2006). The last step is the involution phase in which oxytocin stimulates the uterus to deliver the foetus and placenta (Vannuccini et al., 2016).

1.4 Endocrinology of Steroid hormones, Oxytocin and Prostaglandins during Parturition

1.4.1 Progesterone and Oestrogen

The Corpus luteum is the source of progesterone until seven weeks of pregnancy until the placenta takes over the function at 9 weeks or pregnancy (Kota et al., 2013).

Progesterone and oestrogen play essential roles in both maintenance and initialisation of parturition. Progesterone supports pregnancy by promoting uterine quiescence, while oestrogen stimulates parturition by activating genes that stimulate myometrial contractility and excitability (Challis et al., 2011; Mesiano et al., 2002). The process of parturition in mammals begins with progesterone withdrawal, which leads to decreased circulating progesterone and increased levels of oestrogen (Liggins et al., 1973). However, in the human and higher primates, maternal progesterone and oestrogen are high during pregnancy and remain high during labour (Mesiano et al., 2002; Walsh et al., 1984). A recent study conducted by Muir et al. (2016) identified that the level of progesterone in maternally obese rats is significantly increased at term, but decrease dramatically to similar concentrations observed in control rats in established labour. This evidence suggests obesity may delay the fall in progesterone.

The randomized double-blind, placebo-controlled study with 142 pregnant women who were at high risk of preterm birth reported that administration of progesterone through vaginal suppository significantly reduces preterm birth 2 fold compared to the placebo group (Da Fonseca et al., 2003). Progesterone induces genomic and non-genomic action by activating different receptors. Garfield et al., (2015) proved that the uterus from a pregnant rat supplemented with progesterone in the presence of oestrogen has lower gap junction number compared to progesterone exposure alone and this hormonal environment potentially increases relaxation of the myometrium and decreases the risk of preterm labour. Indeed, many studies provide evidence that progesterone stimulates uterine NO synthetase, which is a major factor in uterine quiescence. Moreover, from the same review of studies progesterone was shown to decrease the regulation of prostaglandin production, calcium channels, oestrogen

receptor expression, oxytocin receptor and interleukin synthase which are associated with inhibition of uterine contractility (Vannucini et al., 2016).

However, there is evidence to suggest that increasing oestrogen in the maternal circulation correlates positively with increasing gestational age (Kote et al., 2013). In the cytotrophoblast, the enzyme CYP450 aromatase converts androstenedione to oestrone and oestradiol using NADPH as a cofactor. Oestradiol is the major active form of oestrogen. At at term pregnancy, the enzyme 17β-HSD located in the foetal membrane allows more oestradiol to access the oestrogen receptor and stimulates prostaglandin release and initiates parturition (Smith, 2001). Oestradiol synthesis is outlined in **Figure 1.1**.

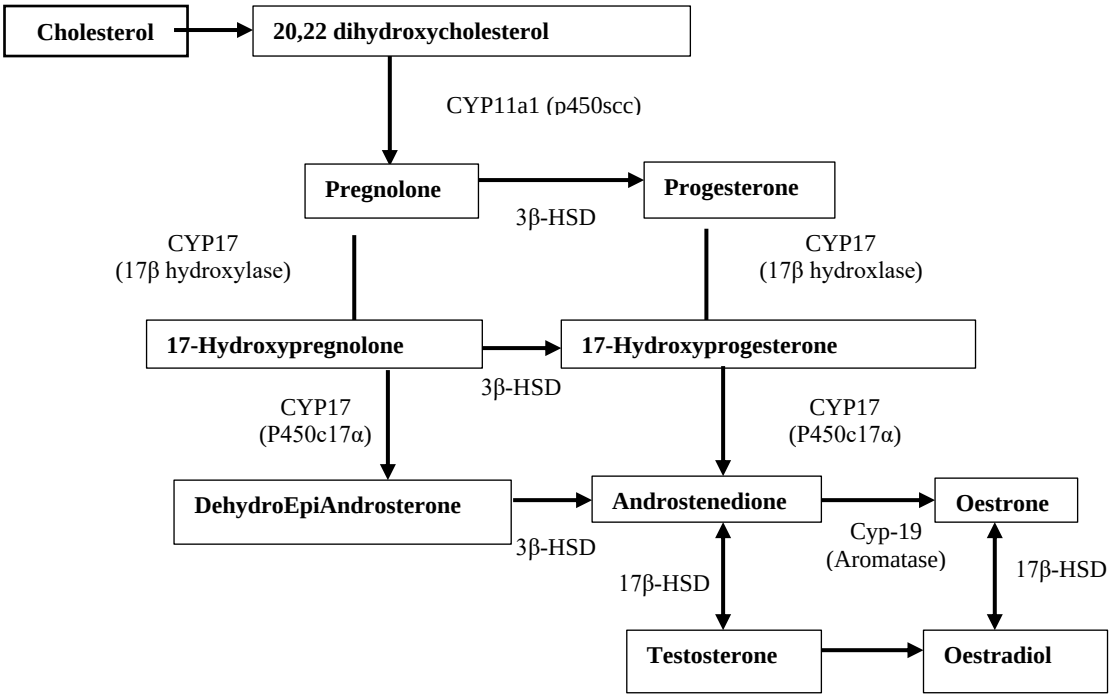

Figure 1.1 Pathway of Oestradiol Synthesis involved the enzymes involved in steroid synthesis and regulated by Thiazolidinediones (TZDs). The 3β-HSD : 3β-hydroxysteroid dehydrogenase. Adapted from Froment et al., (2006).

Many studies stated that Oestrogen not only directly initiates myometrial contractions but also stimulates oxytocin receptor expression and up-regulates enzymes responsible for muscle contraction such as MLCK and calmodulin. Furthermore, oestrogen enhances expression of protein and mRNA for CX-43 and

coordinates gap junction formation in the myometrium (Challis et al., 2000; Kamel, 2010) and controls cervical ripening through the induction of collagenase and elastase (Andersson et al., 2008).

1.4.2 Oxytocin

Oxytocin is a crucial hormone that is involved in 3 phases of parturition: the activation phase, the stimulation phase and the involution phase (Vannuccini et al., 2016). Some published studies reported that oxytocin is a crucial hormone to stimulate myometrial contractions at term labour in both human and animal studies (Arrowsmith et al., 2012; Arthur et al., 2007; Blanks and Thornton, 2003; Morrison et al., 1996).

It has been reported in the literature that the paraventricular and supraoptic nuclei in the hypothalamus generate and secrete oxytocin then it travels to the posterior pituitary through the hypothalamic-hypophysial tract. After oxytocin arrives in the posterior pituitary, it then accesses the systemic blood supply, which delivers oxytocin to oxytocin receptors in the myometrial cells. The oxytocin receptor belongs to the rhodopsin class G-proteins that couple with phospholipase C (PLC) which control the hydrolysis of phosphoinositide-bis-phosphate (PIP_2) into inositol triphosphate (IP3) and Diacylglycerol (DAG) which IP_3 mobilizes calcium from sarcoplasmic reticulum and binds to myosin light kinases resulting in smooth muscle contraction (Arrowsmith, Wray, 2016).

The other mechanism whereby oxytocin induces uterine contractions is associated with prostaglandin synthesis in the decidua. Oxytocin stimulates prostaglandin type-2 synthesis via increased hydrolysis of phosphoinositide to DAG and inositol phosphatase with subsequent release of arachidonic acid from DAG (Flint et al., 1986). Many studies also proved that oxytocin stimulates

myometrial contractions through prostaglandin synthesis, including PGE_2 and $PGF_{2\alpha}$, and also activates the oxytocin receptor which increases intracellular Ca^{2+} in animal cell studies (Alexander and Melvyn, 1993; Arthur et al., 2007; Soloff et al., 2000). This theory is supported by Alexander and Melvyn (1993) who reported a 100-fold increase in PGE_2 synthesis after oxytocin stimulation in amnion cells from rabbits. Furthermore it has been established in both cows and sheep that levels of oxytocin correlate with the levels of oxytocin receptor, and increases in $PGF_{2\alpha}$ synthesis during parturition to increase uterine contractions (Blanks and Thornton, 2003; Fuchs et al., 1996). The diagram of the effects of oxytocin in Prostaglandin synthesis can be seen in Figure 1.2.

1.4.3 Prostaglandin

Prostaglandins are not stored within cells but synthesized in fetal membranes (amnion and chorion), decidua and immediately secreted and act to ripen the cervix, change membrane structure and contract the myometrium (Gibb, 1998). Prostaglandins are bioactive lipid compounds derived from a 20-carbon fatty acid that has an autocrine or paracrine function by binding to specific G-protein-coupled receptors (GPRs), that play an important role in controlling and regulating hormones and numerous second messenger pathways. Furthermore, the action of GPRs is to generate various soluble second messenger such as cAMP and Ca^{2+} leading to activation of intracellular signals and gene transcription related with parturition (Jabbour and Sales, 2004). The study shows that stimulated women foetal membranes with PGE_2 increases MMP-9 production that associated with membrane rupture at term labour (Mc Laren et al., 2000).

Arachidonic acid (AA) is present in various cellular phospholipids, mostly in an esterified form, and the generation of free arachidonic acid for

prostaglandin synthesis is a rate-limiting step in eicosanoid synthesis (Balsindea et al., 2002). Free arachidonic acid release from membrane phospholipids such as phosphatidylinositol and phosphatidylethanolamine, is through the direct action of the enzyme phospholipase A_2 (PLA_2) using an acyl hydrolase stimulation from calcium. The oxytocin-stimulated intracellular Ca^{2+} transients are mediated via Gq and the specific MAPK that catalyses the phosphorylation of cPLA2. In terms of direct activation of arachidonic acid as a substrate for prostaglandin synthesis, the activation of AA is also indirect via the coordination of phospholipase C (PLC) with diacylglyceride lipase and oxytocin (Smith et al., 1991b). The free AA released is then either immediately re-esterified or metabolized to prostaglandin and thromboxane by cyclooxygenase (COX). The three isoforms of COX are l identified as cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and cyclooxygenase-3 (COX-3). COX-2 is a substrate for prostaglandin $PGF_{2\alpha}$ and PGE_2 production (Olson, 2003). The schematic diagram of prostaglandin type 2 synthesis is shown in **Figure 1.2.**

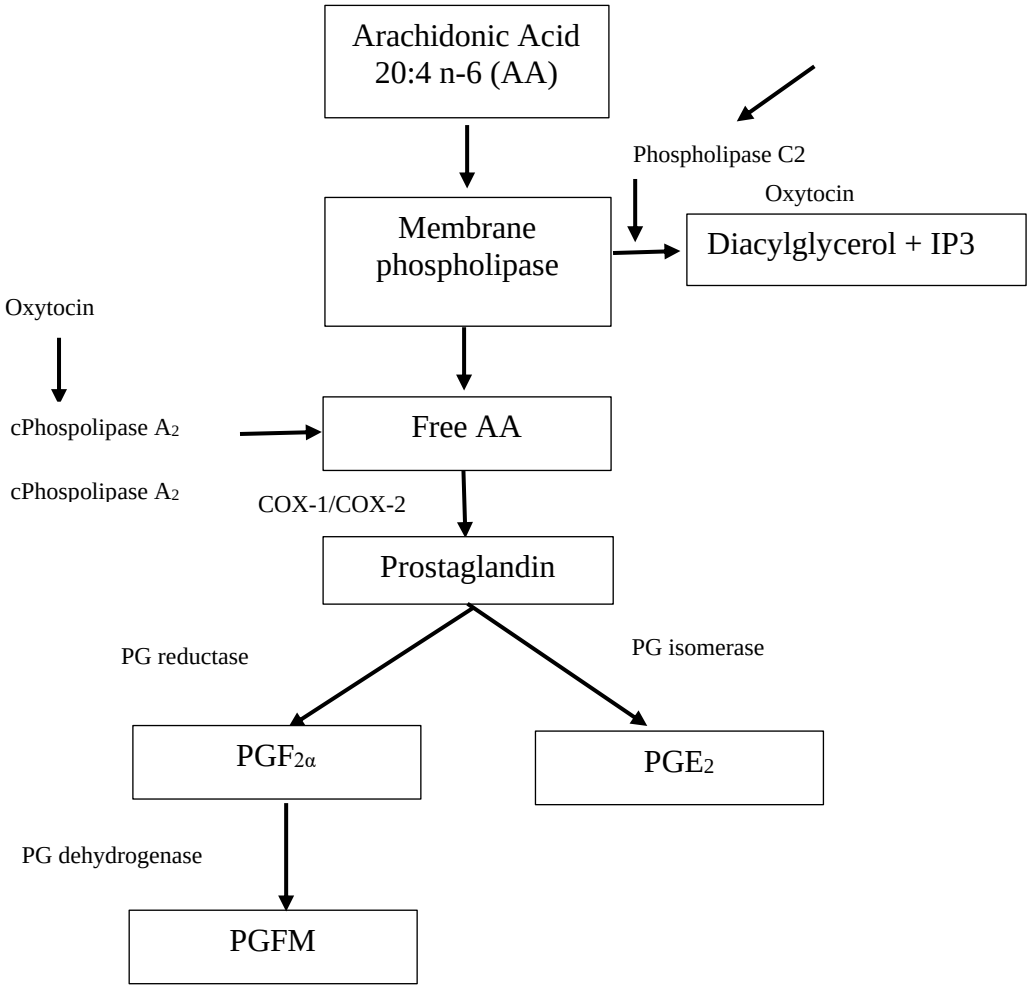

Figure 1.2 Schematic diagram of the role of Arachidonic acid (AA), oxytocin, prostaglandin enzymes in prostaglandin type-2 ($PGF_{2\alpha,}$ PGE_2) synthesis. Adapted from Abayasekara & Wathes (1999)

The action of prostaglandin is dependent on the specific receptor. A Review conducted by Coleman et al., (1994) reported that PGE_2 could both stimulate and inhibit contractions while the receptor for $PGF_{2\alpha}$ known as FP receptor stimulates myometriual contractions through the FP receptor by elevated intracellular free calcium. This theory is supported by Arulkumaran et al., (2012) that provided evidence that administration of PGE_2 increased uterine contractions, whereas adding the EP_3 antagonist L798106 inhibited both spontaneous and PGE_2-induced contractions in human pregnant myometrium at term *ex-vivo*. However, Hinton et al., (2010) showed decreased protein expression of the PGE

EP_4 receptor in the pregnant rat cervix following progesterone supplementation in order to delay labour. This data suggests that PGE_2 can stimulate contractions via EP_3 but also can inhibit contractions through the EP_4 receptor.

Furthermore, many studies show evidence that regulation of prostaglandins is also dependent on glucocorticoids, inflammatory cytokines, progesterone and oestrogen (Challis et al., 1997). There is a lot of evidence to suggest positive feedback between prostaglandins and inflammatory cytokine release by infiltrating leukocytes (Phillips et al., 2014). This theory is supported by Rauk and Chiao (2000) that showed IL-1 stimulation of human uterine smooth muscle cells increased prostaglandin production by enhanced protein and mRNA expression of COX-2. The synthesis and release of prostaglandin type-2 is associated with oestrogen production during parturition since whittle et al., (2000) reported increased plasma PGFM in oestrogen stimulated pregnant cow at term and it is associated with increased COX-2 mRNA in the placenta and intercotyledonary endometrium. It is important to note that prostaglandin analysis is conducted based on 13,14-dihidro-15-keto-Prostaglandin F_2-alpha (PGFM) and PGE_2 metabolites or 13,14-dihidro-15-keto-Prostaglandin E_2 (PGEM) levels in peripheral plasma, as an indicator of consistent uterine prostaglandin production (Heuwieser et al., 1993).

1.5 Molecular intracellular signalling pathways of the activation until involution stage of parturition

CAPs, Oxytocin, Peroxisome Proliferator Activation Receptor (PPARs, Aromatase and Protein Kinase C (PKC) are important to activation pathways of parturition. Inhibition of PPARs contribute to activate Oestrogen through increased aromatase activation that can increase the CAPs activation especially CX-43 and COX-2. Pro Inflammation agent and Oxytocin increased prostaglandin type two synthesis

through COX-2 acivation that very essential during stimulation until involution stage of parturition (Vannucini et al., 2016).

1.5.1 Inflammatory cytokines

Many studies have found the third trimester of pregnancy to be associated with an increase in inflammatory activity, including increased protein and mRNA expression of inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α in the human uterus, cervix and placenta (Dubicke et al., 2010; Mouzan and Guerra-Millo, 2006). Review studies highlight that increased cytokine expression plays an important role in promoting the expression of both contractile-associated proteins (CAPs) such as Oxytocin receptor (OXTR), Connexin-43 (CX-43) and metalloproteinases (MMPs) in both human isolated and cell pregnant myometrium. Cytokine expression could therefore be responsible for triggering the final aspects of parturition, including membrane rupture, uterine contraction and cervical maturation (Sivarajasingam et al., 2021). In addition, Chen et al. (2019) observed that the myometrium of women in term labour had higher mRNA levels of IL-1β, IL-6, TNF-α and CAPs when compared with the myometrium of non-term women.

Tribe et al., (2003) found evidence that exposure to IL-1β (10 ng/ml) for 24 hours increases the protein expression of sarcoplasmic reticulum calcium ATPase (SERCA) 2b, which is responsible for refilling the sarcoplasmic reticulum with Ca^{2+}. Moreover, an increase in this protein is associated with enhanced basal calcium entry and the initiation of spontaneous calcium transients, which have the potential to increase direct myometrium contractions in human myometrial smooth muscle cells. In addition, IL-1β plays an important role in increasing myometrial contractions through stimulating arachidonic acid release and increasing COX-2 activity and prostaglandin production in cultured cervix fibroblast cells (Sato et al., 2001) and human myometrium cells (Peltier,

2003; Pollard and Mitchell, 1996). Another inflammatory cytokine, namely tumour necrosis factor-α (TNF-α), has the potential to promote labour through increasing prostaglandin production. This was observed by Alexander et al. (2012), who reported TNF-α treatment to increase the protein levels of mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (Nf-kB) in human uterine smooth muscle cells. The inhibition of protein MAPKs and NF-kB reduces the activity of COX-2 and, therefore, has the potential to decrease contractions. This suggests that inflammatory cytokines affect myometrium contractions through downstream signalling. Another study noted a fivefold increase in the number of mRNA oxytocin receptors (OXTRs) in primary human myometrium cultures after four hours of treatment with IL-6 (Rauk et al., 2001).

1.5.2 Connexin-43 (CX-43)

Connexin-43 (CX-43) is molecule that regulates the direct communication properties of the gap junctions between adjacent cells. More specifically, CX-43 is inserted into the membrane of the ER after transcription and translation, where folding and post-translational modification occur. Connexins (CXs) are regulated by post-translational modifications such as phosphorylation. In the endoplasmic reticulum (ER) and Golgi/trans-Golgi, CXs are assembled into hexamers known as hemichannels (HC) which facilitate intracellular cell communication (Puebla et al., 2017).

Oligomerization of connexins usually occurs during the transition from the ER to the Golgi apparatus and, later, in the trans-Golgi apparatus network (TGN). However, although most connexins are oligomerised in the ER, the late oligomerisation of CX-43 has been evolutionary established in order to control heteromeric hemichannel formation alongside other connexin isoforms, such as CX-40 and CX45 (Epifantseva and Shaw,

2018). The mechanism by which newly translated oligomerised proteins are delivered to their target location involves them freely diffusing within the lipid bilayer to reach the final functional destination. Alternatively, the connexins or hemichannels exit the Golgi and then take a more direct route to particular membrane subdomains such as the cell–cell border or intercalated discs (IDs). Non-sarcomeric actins (filamentous or F-actins), which consist of β and γ forms, serve as important participants in the targeted delivery of CX-43, since F-actins provide a pool of ready-formed hemichannels available to deliver to the IDs and act as vesicle transporters along the microtubules to the target location within the cell membrane (Epifantseva and Shaw, 2018).

CX-43 has been shown to be the dominant connexin in both rodent and human myometrial cells, in addition to in cultured human myometrial cells (Hendrix et al., 1992). It has also been established that the single gap junction plaque at-term human myometrial smooth muscle cells contains three different connexins, namely CX-40, CX-43 and CX-45, which suggests the important role of connexin in modulating the communication properties of the smooth muscle cells during human pregnancy (Kilarski et al., 2001). A review study found that CX-43 allows the cells that facilitate the ionic coupling of myometrial cells to coordinate contractions (Kidder and Winterhager, 2015). The study further reported that oestradiol increases the mRNA and protein expression of CX-43, while progestin steadily decreases the mRNA and protein expression of CX-43 in human myometrium cells (Firestone and Kapadia, 2012). Moreover, an experimental study conducted by Doring et al. (2006) determined that the deletion of CX-43 in mice myometrial smooth muscle cells increased the time to labour, although delivery still occurred because other connexins (CX-40, CX-46 and CX-26) were expressed and compensated for the loss of CX-43.

1.5.3 Caveolin-1

Caveolins form in the main structure of the caveolae and lipid rafts, which feature 50- to 90-nm flask-shaped invaginations and appear in the smooth muscle membrane (Taggart, 2001). Lipid rafts are plasma membrane microdomains enriched with cholesterol and sphingolipids, which are involved in the lateral compartmentalisation of the molecules at the cell surface. Lipid rafts contain a mixture of membrane proteins, including caveolins (Orkin, 2015). The caveolins are scaffolding proteins that facilitate the assembly of signalling complexes and act as message centres for the integration of signal transducers. Various studies have found that caveolins bind with G-protein-coupled receptors, protein kinase C, nitric oxide synthase, PKC-α, RhoA and receptor-dependent tyrosine kinases, with such binding being suggested to alter the activity of signalling molecules (Okamoto et al., 1998). This theory is supported by the work of Je et al. (2004), who found evidence that loading caveolin scaffolding domain peptides in the vascular smooth muscle cells of ferrets disrupted phorbol ester protein kinase C activation and increased both ERK1/2 activation and CaD phosphorylation. The phosphorylation of CaD can stimulate actin for interaction with myosin, thereby leading to increased contractions. This suggests that Cav-1 might plays an important role in the coordination of signalling, leading to the regulation of the contractility of the smooth muscle.

Caveolins are abundant in the smooth muscle, and they serve as a location for calcium storage and entry. Sugi et al. (1982) found that pyroantimonate precipitates calcium accumulation in the caveolae of the smooth muscle under quiescent conditions, while Daniel et al. (2009) reported that the absence of caveolae in the interstitial cells of Cajal (ICC) inhibits its ability to maintain the frequency of contractions in a calcium-free medium by reducing the recycling of calcium from the caveolar plasma membrane to the

sarcoplasmic reticulum (SR) when the calcium store is initially full. In addition, Shaul et al. (1995) identified a number of critical molecules involved in calcium transport, such as the inositol 1,4,5-triphosphate (IP3) receptors, calcium ATPase, calmodulin and endothelial nitric oxidase synthase (eNOS), which are all located in the caveolae and serve as key signalling molecules in endothelial cells. Norman et al., (1999) also noted that protein expression of eNOS increased in the human preterm pregnant myometrium and potentially induced myometrial contractions.

Furthermore, a study conducted by Nuno et al., (2012) reported that Cav-1 inhibits the redistribution of proteins that play an important role as mediators of smooth muscle contractility, such as protein Ras homologue protein A (RhoA) in vascular rat and Rho- associated protein kinase (ROK) (Aguilar and Mitchell, 2010). One review reported that ROK is crucial when it comes to inducing the release of Ca^{2+} from the SR, which indirectly renders the cell more excitable (Lee et al., 2001b; Taggart, 2001). Moreover, Taggart (1999) also noted that the translocation of both RhoA and ROK from the cytoplasm to the plasma membrane increases the Ca^{2+}-sensitised contractions in the uterine smooth muscle. However, another study found the absence of Cav-1 to have no effect on the amplitude of contractions, but it decrease the rate of calcium release from the SR and decreased the ability of Cav-1 to handle the Ca^{2+} channel in mouse intestine smooth muscle (Daniel et al., 2009). This mechanism could also occur in the myometrium, where decreased Cav-1 expression might suppress the release of Ca^{2+} from the SR to the intracellular myometrium cells and, therefore, potentially affect myometrial contractions.

1.5.4 Cyclooxygenase-2 (COX-2)

Cyclooxygenase-2 (COX-2) is an enzyme that plays an important role in prostaglandin type-2 synthesis (Simmons et al., 2004; Wang et al., 2016). According to review study conducted by Ricciotti and Fitzgerald (2011) prostaglandin type 2 are generated from arachidonic acid by the action of COX-2. Moreover, COX-2 is not found in the majority of tissues; rather, it is largely found in macrophage and inflammatory tissues that have been exposed to cytokines, mitogens and growth factors. Prior studies have reported that COX-2 is expressed in the uterus and embryonic membrane, as well as being highly abundant in the amnion, although it is rare in the placenta (Dunn-Albanese et al., 2004; Slater et al., 1995).

There is much evidence to suggest an association between the upregulation of protein and mRNA for COX-2 and the increased expression of pro-inflammatory cytokines, including the interleukins (IL-1 and IL-2) and TNF-α in human and animal studies (Abdalla et al., 2005; Ackerman et al., 2005b; Gross et al., 2000; Lundin-Schiller and Mitchell, 1991; Rauk and Chio, 2000). Studies have also shown that stimulating human myometrial cells with IL-1 β increases the protein expression of COX-2 through activating both Nf-kB and protein kinase C (PKC) (Wouters et al., 2014). Wouters et al. (2014) also found that stimulating myometrial cells with oxytocin and Epidermal Growth Factor (EGF) increases the protein expression of COX-2 via PKC signalling and Extracellular signal-regulated kinases (ERK) in the primary human myometrium cells. Furthermore, some studies have suggested the increased mRNA expression of COX-2 achieved through PPARδ/α inhibition to be positively correlated with increased levels of $PGF_{2\alpha}$ and PGE_2 in cells of the bovine myometrium (MacLaren et al., 2006). Oxytocin, nuclear protein receptors (e.g. peroxisome proliferator-activated receptors), PKC, Nf-kB, EGF and ERK have the potential to induce the transcription of a large number of

inflammatory genes, including COX-2. The underlying COX-2 signalling pathways activated by oxytocin, ERK, IL-1 β and peroxisome proliferator-activated receptors are shown in **Figure 1.3**.

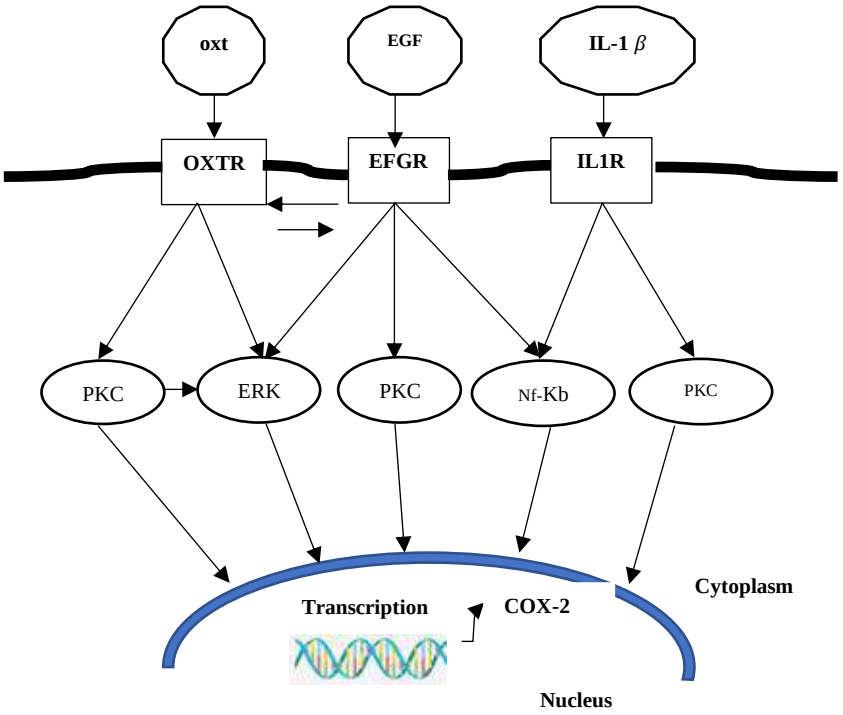

Figure 1.3 Underlying signalling pathways activated by Oxytocin receptor (OXT), Epidermal Growth Factor (EGF) and Interleukin-1β (IL-1β) to stimulate Cyclooxygenase-2 (COX-2) expression in human myometrial cells adapted from Wouters et., (2004).

Prior studies have found the inhibition of COX-2 to be associated with uterine relaxation *in vitro* (Slattery et al., 2001). Gross et al. (2000) observed that the administration of lipopolysaccharide (LPS), as an inflammation stimulator, to the uterus of mice can increase the mRNA expression of COX-2 and $PGF_2\alpha$ in the uterus and potentially increase the risk of preterm birth. Moreover, it has been reported that oestradiol increases the intrauterine mRNA and protein expression of COX-2 and also upregulates maternal plasma $PGF_2\alpha$ production, thereby causing premature delivery in pregnant sheep (Wu et al., 2004).

1.5.5 Peroxisome proliferator-activated receptors

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligands that activate transcription factors. There are three major isoforms of PPARs, namely PPAR-α, PPAR-δ and PPAR-γ (MacLaren et al., 2006). These three isoforms have distinct tissue expression patterns and ligand sensitivities, and they regulate the expression of the interactive gene networks that govern energy and metabolic homeostasis, glucose utilisation and cell growth and differentiation. The dysregulation of PPAR functionality directly affects the aetiology and pathogenesis of disorders such as obesity (Patel et al., 2004).

The structure of a PPAR consists of a DNA-binding domain in the N-terminus and a ligand-binding domain in the C-terminus. Following interactions with agonists, PPARs are translocated from the cytoplasm to the nucleus, where the PPARs heterodimerise with RXR and then regulate gene transcription (Grygiel-Gorniak, 2014). Moreover, a review study conducted by Grygiel-Gorniak (2014) revealed that the gene targets of PPAR-α are the acyl-Co-A oxidases responsible for the β-oxidation pathways, sterol 12-hydroxylase (CYP8B1), fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36), lipoprotein lipase and apolipoprotein A-1. The target genes of both PPARδ and PPAR β are associated with lipid uptake, metabolism and efflux (which are repressed by PPARs), while the gene targets of PPAR γ are fatty acid-binding protein (aP2), FATP and CD-36.

PPARs are activated by the binding of natural ligands such as polyunsaturated fatty acids (PUFAs) or synthetic ligands such as thiazolidinediones (TZDs). In addition, PPARs are expressed in various organs, including the hypothalamus, pituitary, ovary and uterus (Froment et al., 2006). It has been established that the placenta represents an important expression site for the three PPAR isoforms in gestating mammals (Fournier et al., 2007b). Borel et al. (2008) determined that both the mRNA and protein expression

of PPARs occurs in the placenta, amnion, chorion and amnion-epithelial cells. Numerous studies have found that PPARs activation play an important role during the activation and stimulation phases of pregnancy through altering both prostaglandin and pro-inflammatory cytokine synthesis as well as oestrogen production in the placenta (Borel et al., 2008; Fournier et al., 2007a; Matsuda et al., 2013).

The activation of PPARs is known to be affected by pro-inflammatory cytokines, prostaglandins, fat-including cholesterol and PUFAs (Ackerman et al., 2005a; Ebrahimi et al., 2015; Sikder et al., 2018; Stienstra et al., 2007; Varga et al., 2011; Zuniga et al., 2011). The lipids in the human chorion and amnion are enriched with arachidonic acid, which is a precursor for prostaglandin type-2 synthesis. Moreover, the majority of the arachidonic acid found in foetal membranes can be easily converted into prostaglandin D_2 (PGD_2) and prostaglandin J2 (PGJ2), which are co-expressed with PPAR-γ in the placenta. PGJ2 is a potential selective activator of PPAR-α and PPAR-γ. Arachidonic acid is not the only unsaturated fatty acid that affects PPAR synthesis. Indeed, some studies have reported omega-3 fatty acids such as Eicosapentaenoic Acid (EPA), Decosahexaenoic acid (DHA) and Alpha Linolenic Acid (ALA) to induce the synthesis of PPARs via the inhibition of pro-inflammatory markers such as IL-6 and Nf-kB in both humans and animals (Ebrahimi et al., 2015; Li et al., 2005; Zuniga et al., 2011).

The mechanism by which PPARs suppress pro-inflammatory markers such as IL-1, IL-6 and Nf-kB plays an important role in uterine quiescence during pregnancy (Borel et al., 2008). However, immunohistochemical analysis suggests that the PPAR-γ levels reduce once active labour commences, coincidental with a relative increase in the expression of COX-2 in the intrauterine tissue of humans who deliver via caesarean section (Dunn-Albanese, 2004). The mechanisms by which PPAR-γ initiates and suppresses contractions are shown in **Figure 1.4**.

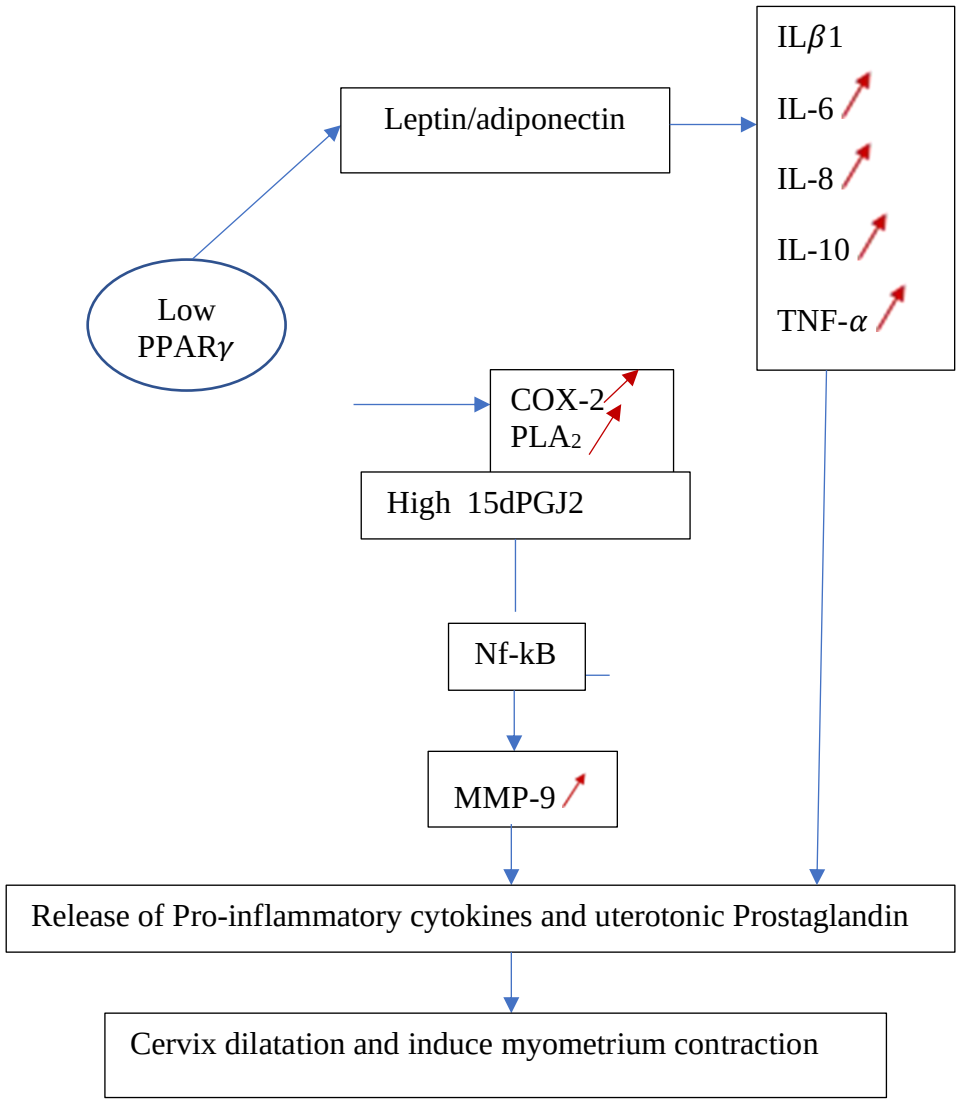

Figure 1.4 The role of PPAR-γ in the stimulation of contractions during pregnancy. Low expression of PPAR-γ can stimulate contraction at term labour through COX-2 and pro-inflammatory cytokine activation. PLA2 : Phospholipase A2,NFkB : Nuclear Factor Kappa B, 15dPGJ2 : 15-Deoxy-Δ12, 14-prostaglandin J2, IL-8 : Interleukin-8, IL-10 : Interleukin 10, IL-6 : Interleukin-6, IL1β : Interleukin-1 β adapted from (Borel et al., 2008).

The evidence of study conducted by Han et al., (2003) shows that PPAR-γ regulated pro-inflammation molecules whereas PPAR-γ ligands increased mRNA of PPAR-γ but down regulated mRNA COX-2. From the same study also proved that PPAR-γ ligands suppressed the binding activities of Nf-kB in human cervical cancer

cells. Furthermore, down regulation of PPAR-γ expression decreased gene of MMP9, but upregulated IL-6, IL-1β and TNF-α invitro and in vivo in placenta pregnant rat cell and tissue (Li et al., 2022).

1.5.6 Protein Kinase C (PKC)

PKC contributes to the activation of parturition by modulating either solely the lipid signals or both the lipid and Ca^{2+} signals (Lipp and Reither, 2011). PKC can be classified into three types depending on the cofactor requirements. The first type is conventional PKC (cPKC), which has isoforms known as PKCα, PKCβ1, PKCβ2 and PKCγ. The cPKC is diacylglycerol (DAG)-sensitive and Ca^{2+}-responsive. The second type is novel PKC (nPKC), which has isoforms known as PRKC,δ, ε,η,θ and μ. nPKC is DAG-sensitive but Ca^{2+}-insensitive. The third type is atypical PKC (aPKC), which has isoforms known as PKC ζ and λ. aPKC is regulated by phospholipidic mediator products such as phosphatidylinositol 3,4,5-triphosphate (Eude-Le Parco et al., 2007).

The activation of PKC is not only induced by diacylglycerol and calcium, as it can also be increased by IP-3 via growth factors, cytokines, hormones and oxidative stress. Increased IP-3 synthesis has been reported to both increase Ca^{2+} sensitisation and induce vascular smooth muscle contraction via the activation of MLCK (**Figure 2.4**) (Ringvold and Khalil, 2017). Furthermore, although PKC is located in the cytoplasm, the generation of DAG can increase the membrane-bound fraction of PKC via translocation. The membrane-binding mechanism of PKC requires both calcium and phosphatidylserine. Under these conditions, the enzyme can interact with the substrate, albeit with only very low catalytic efficiency (Khan et al., 1991). Ringvold and Khalil (2017) reported that PKC could cause the phosphorylation of CPI-17, which inhibits protein MLC phosphatase and increases MLC phosphorylation that plays an important role in increasing smooth muscle contractions. Moreover, PKC activation also induces

the phosphorylation of the actin-binding protein calponin (CaP), which allows more actin to bind myosin and, therefore, enhances the vascular contraction process (Ringvold and Khalil, 2017). It is possible that the mechanism behind the PKC-induced contraction of smooth muscle also occurs in the myometrium. The contraction pathways activated by PKC are shown in **Figure 1.5**.

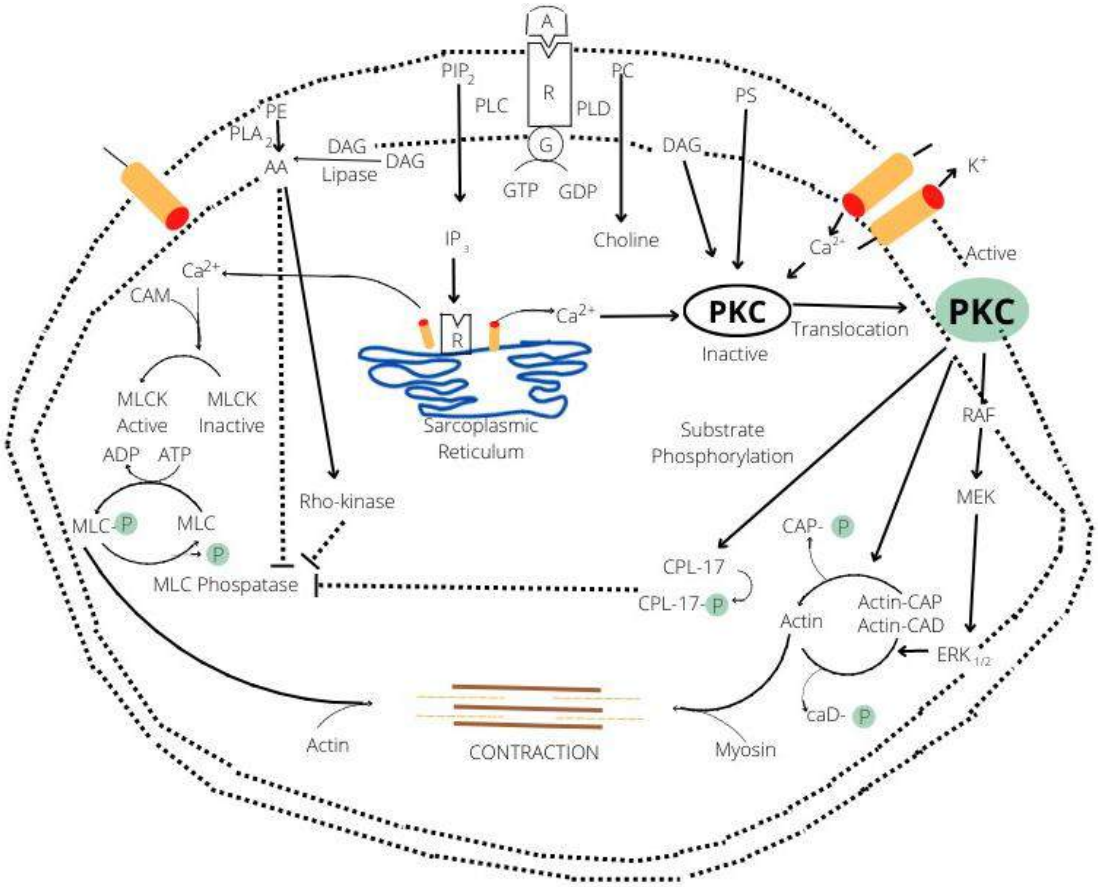

Figure 1.5 The Pathways of contraction stimulation via Protein Kinase C (PKC) activity in Vascular Smooth Muscle. DAG: Diacyglycerol, MLC : myosin light chain, PLD : phospholipase D, PLC : Phosphatidylcholine, PLA_2 : Phospholipase A2. AA : Arachidonic acidMLCK : Myosin L Chain Kinase, PKC : Protein Kinase C, PE: Phosphatidylethanolamine, RhoA ; Rho A Kinase, adapted from (Ringvold and Khalil, 2017)

Hyperglycaemia also activates the *de novo* pathways of diacylglycerol synthesis, thereby resulting in PKC activation (Eude-Le Parco et al., 2007; Meier and King, 2000). Moderate hyperglycaemia can activate the p38 MAP kinase pathway, which has been shown to stimulate cPLA2, a key enzyme associated with arachidonic acid production via the PKC-δ-isoform-dependent pathway. An increase in cPLA2 is

known to be associated with prostaglandin type-2 production in cultured glomerular mesangial cells (GMCs) (Williams and Schrier, 1993).

PKCδ has been shown to increase the contractile activity of human myometrial biopsies *ex vivo* (Eude-Le Parco et al., 2007). In addition, it has been reported that PKCδ is an essential component of the TNFα and IL-1β pathways for controlling Nf-kB activation in both animal and *in vitro* studies (Diaz-Mecol et al., 1994; Leitges et al., 2001). Duggan et al. (2007) found evidence to suggest that atypical PKC isozymes mediate the NF-kB transcriptional activation required for IL-1β to increase prostaglandin type-2 production and, potentially, myometrial contraction. Moreover, oxytocin-stimulated phosphatidylinositol 4,5 bisphosphate (Ptdlns 4,5-P2) is associated with the generation of two second messengers, namely inositol 1,4,5-triphosphate (Ins 1,4,5-P3) and sn-1,2-iacylglycerol (DAG), which stimulate the release of calcium from the intracellular store, thereby raising the cytosolic calcium concentration, suggesting the combination of DAG and calcium to be the main physiological activator of PKC (Bemdge and Irvine, 1989).

Interestingly, PKC activation might decreased the protein expression of CX-43 and CX-43 trafficking and inhibit both gap junction assembly and channel gating in the vascular cell and tissues (Lampe and Lau, 2004). This mechanism possibly occurs in the uterus. Furthermore, effective parturition requires the synthesis and assembly of CX-43, meanwhile the gap junction disassembly are associated with unsynchronised contractions in maternal obesity (Hendrix et al., 1992; Muir et al., 2016). Moreover, Garfield and Hayashi (1981) reported a significant correlation between increased cervical dilatation, increased contraction frequency and increased gap junction area in the myometrium during labour (Garfield and Hayashi, 1981).

PKC activator and inhibitor drugs have been used in some studies to investigate the role of PKC in altering smooth muscle contractions *ex vivo* (Massenavette et al., 2017; Rybin et al., 2008). For instance, phorbol-12-myristate 13 acetate (PMA) has been found to increase MMP regulation by means of PKC activation (mainly cPKC and nPKC), although this induction can be counteracted by concurrent exposure to a PKC inhibitor (Go6983) (Park et al., 2000). In addition, a study reported that Go9683 inhibited PKCα, PKCβ and PKCδ and also induced the marked inhibition of phorbol 12,13-butyrate (PDBu), a PKC activator known to stimulate myometrial contractions (Ozaki et al., 2003).

1.6 Fatty acids

1.6.1 Fatty acid signalling

Fatty acids such as saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFAs) are important substrates for the oxidation and production of cellular energy, including the precursor molecules for all lipid classes and biological membranes. Yet, free fatty acids alter gene transcription activation, post-transcriptional protein modification and the direct modulation of enzyme activity as co-activators (Pacakova and Cahova, 2015).

Fatty acids also affect membrane fluidity. More specifically, membrane fluidity increases as the level of unsaturated fatty acid increases (SFA<MUFA<n-6 PUFA<n-3 PUFA). This was noted by Prasad et al. (2010), who reported the higher membrane fluidity of mice thymocytes following the administration of 30 μM docosahexaenoic acid (DHA) and 30 μM arachidonic acid (AA) with two hours of incubation time when compared with the administration of 30 μM stearic acid over the same incubation time. An alteration in membrane fluidity has been shown to affect membrane signal

transduction, since increased membrane fluidity enhances the coupling efficiency of G-proteins while decreased membrane fluidity decreases the interaction between B-Ars and G-protein. Among the G-protein-coupled receptors are the free fatty acid receptors FFAR1 and FFAR-4, which are activated by medium- and long-chain fatty acids. The activation of these FFARs might be involved in the intracellular signalling pathway associated with the abundance of protein kinases such as PKC, MAPK and connexin (Poebla et al., 2017). Moreover, caveolae and lipid rafts contain many saturated acids, which means that unsaturated fatty acid incorporation will change their stacking and natural liquid-ordered state. Once incorporated, unsaturated acids can alter their elasticity, compressibility and ion permeability (Packoya and Cahoya, 2015).

Fatty acids can move from the plasma membrane to the cytosol through proteins associated with transmembrane transport, including CD36. CD36 is a fatty acid translocase that exhibits strong binding affinity with a wide range of fatty acids. The regulation of CD36 occurs at the transcriptional and translational levels, and it is mediated by the transcriptional-binding CCAAT/enhancer-binding protein alpha and PPARs. Fatty acids are also strong regulators of the transcription of CD36 in various bovine cells through PPARs (Bionaz et al., 2020).

In addition, fatty acids play an important role in regulating cellular metabolism through acting as agonistic ligands of different nucleus receptors. When activated, these ligands bind to the promoter region of a specific gene and then selectively induce transcription. Among the nuclear transcription factors that can bind with free fatty acids (FFAs) are PPARs and sterol-regulatory element binding proteins (SREBPs). Due to the extremely low solubility of FFAs in cytosol, the translocation of FFAs from the plasma membranes to the intracellular cells occurs. FFAs are taken up into the cells by protein transporters within the plasma membrane and then transported intracellularly

via fatty acid-binding protein (FABP) (Packoya and Cahoya, 2015). This FABP is a membrane-bound protein present in both the mitochondria and plasma membrane that plays an important role to translocate fatty acid within cooperation with CD36 (Bionaz et al., 2020).

1.6.2. Metabolism of Polyunsaturated fatty acid (PUFA)

Polyunsaturated fatty acids (PUFAs) are hydrocarbon chains that terminate in a carboxyl group that has one carbon and multiple double bonds and named according to the number and position of double bonds, Fatty acids usually occur in natural fat and oils as esters, but it also found in plasma as a non-esterified form as a free fatty acid. Some fatty acids do not contain a double bond in the carbon backbone and are known as a saturated fatty acids. Meanwhile, a fatty acid with one or more double bonds at the carbon backbone is called an unsaturated fatty acid. Unsaturated fatty acids are divided into monounsaturated (one double bond), polyunsaturated (PUFAs, two or more double bonds) and eicosanoids (Abayasekara and Wathes, 1999).

PUFAs are generally synthesized by the modification of saturated fatty acid precursors that are products of fatty acid synthase. The desaturase enzymes insert a double bond at a specific carbon atom in the fatty acid chain and fatty acid elongation system elongates the precursors in two-carbon increments (Patterson et al., 2012). Furthermore, desaturases catalyse the introduction of bonds between the carboxylic end of the molecule and pre-existing double bond to introduce further unsaturation into existing PUFA. There are two types of desaturases namely front-end desaturases such as delta-4, delta-5 and delta-6 desaturase and methyl-end desaturases, such as delta-12 and delta-15 desaturases (ω3 desaturases), Front-end desaturases introduce the double bond between the carboxylic end of the molecule and the pre-existing

double bond to generate PUFA. The second type of desaturases are called methyl-end desaturases that add a double bond between the pre-existing double bond and methyl end in fatty acids. However, mammals, including humans, do not contain these methyl-end desaturases for producing essential fatty acids. Therefore, mammals must acquire essential fatty acids such as Linoleic acid (LA) and ALA from foods or nutritional supplements (Nakamura and Nara, 2004).

From **Figure 1.6** we can see that omega-3 and omega-6 PUFAs compete for use of the same desaturase and elongase enzymes to add carbons or double bonds in their chains. The LA and ALA are converted to Gamma Linoleic acid (GLA) and stearidonic acid (SA) respectively, by delta-6 desaturase. The Elongation of very long chain fatty ac-5 (ELOVL 5) enzyme is required to elongate GLA to Dihommo-gamma-linoleic acid (DGLA) and Stearidonic Acid (SA) to eicosatetraenoic acid (ETA) by two-carbon elongation. DGLA is then converted to AA by delta-5-desaturase enzyme or FADS1. AA can be further elongated by ELOVL2 to decosatetraenoic acid (C22:4n-6) or its respective set of eicosanoic acid via COX-2. The synthesis of AA derived eicosanoids is dependent on the DGLA concentration because DGLA competes with AA for COX synthesis. Meanwhile, ELOVL2 also used by Eicosapentaenoic acid (EPA) is to synthesize Decosapentaenoic acid (DPA). Finally, the delta-6-desaturase enzyme is used to convert Decosapentaenoic (DPA) to Decosahexaenoic acid (DHA) (Patterson et al., 2012).

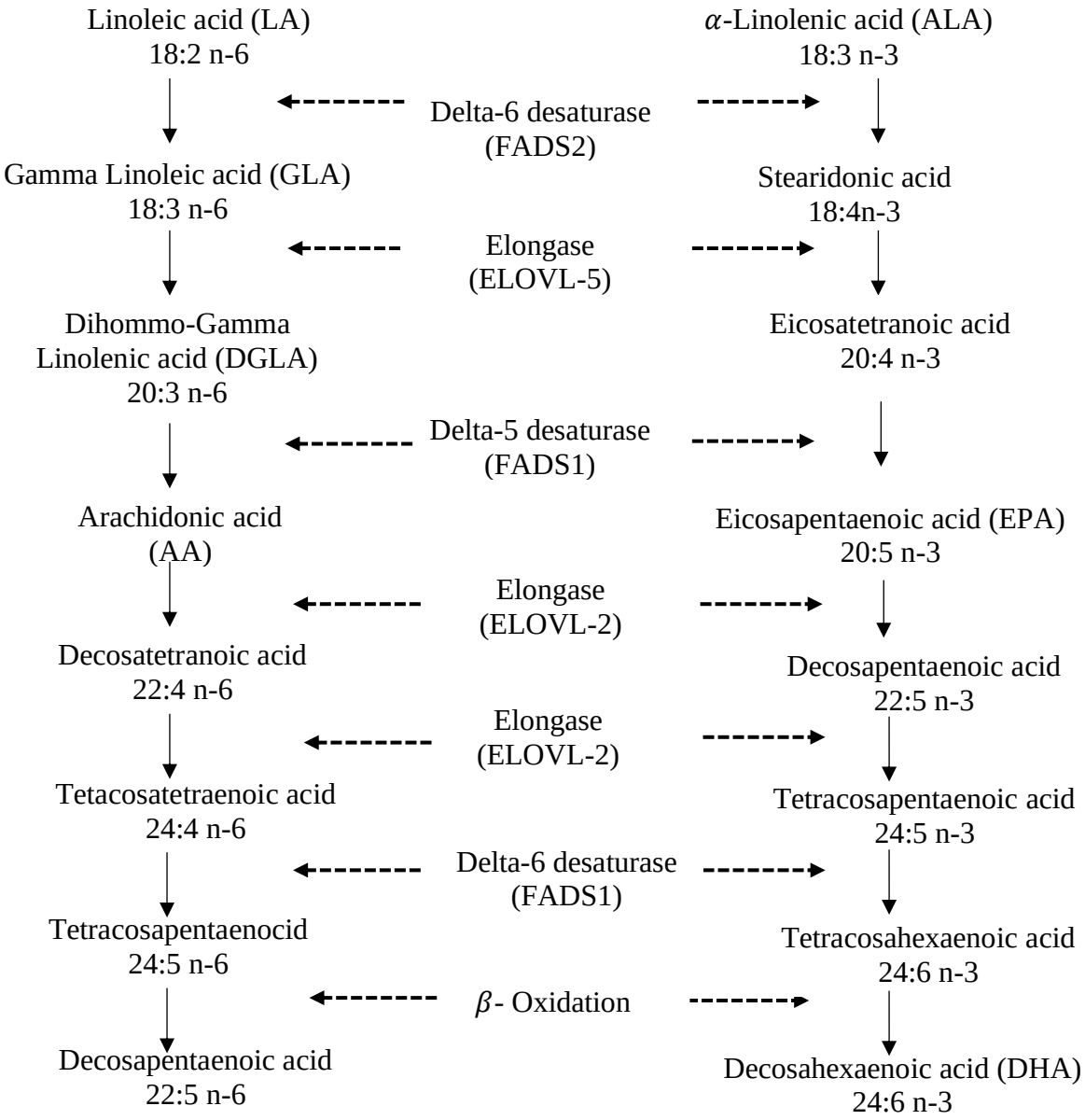

Figure 1.6 The pathways of both omega-6 and omega-3 metabolism to a longer chain of unsaturated fatty acid, involving several enzymes of elongation : Elongation of very long fatty acid-2 (ELOVL-2, desaturation ; Fatty acid desaturase-1 and 2 (FADS-1 and FADS2) and β-oxidation, adapted from Patterson et al., (2012)

1.6.3 Effect of Fatty acids on parturition

1.6.3.1 Effect of PUFAs on prostaglandin synthesis

Prostaglandin, especially the 2-series, including PGE_2 and $PGF_{2\alpha}$, are key signals in parturition. At term, PGE_2 causes cervical dilatation, while $PGF_{2\alpha}$ stimulates

uterine contractions and delivery (Challis et al., 2002a). In mammals, various eicosanoids, including prostaglandins, are produced by the metabolism of omega-6 and omega-3 PUFAs. EPA is an immediate precursor for the 3-series prostaglandin, while AA produces the two-series prostaglandin via the cyclooxygenase pathway and Dihomo-Gamma Linolenic Acid produces the 1-series prostaglandins (Fain et al., 2001). The proportion of fatty acids in the diet affects PUFA composition in the cell membrane, and this will correlate with the level of prostaglandins synthesised since the precursors for each prostaglandin compete for the same enzyme systems for metabolism (Lands, 2015).

There are two crucial enzymes namely delta-6 and delta-5 desaturase that are important for PUFA metabolism to produce prostaglandins. The delta-6 and delta-5 desaturases are rate-limiting to a large number of the substrates, including 18 and 22 carbon fatty acids (Guillou et al., 2004). Thus, some PUFAs are competing with each other to use the enzymes. The DGLA is substrate for synthesis of one series prostaglandins and the n-3 PUFA in the diet has a significant correlation with reducing cyclooxygenase metabolites, COX-2 is the enzyme that induces prostaglandin type-2 synthesis (Kelley et al., 1985; Kirkup et al., 2010; Lands, 2015). Further support that Omega-3 PUFAs regulate the timing of labour is that a diet high in n-3 PUFAs delayed the onset of uterine parturition and increased gestational length in sheep (Szajewska et al., 2006). In contrast, raised dietary LA intake during pregnancy increases 2-series prostaglandin synthesis and potentially increases the risk of preterm birth in ewes (Elmes et al., 2005). Similarly, a higher ratio of n-6:n-3 fatty acid significantly increase AA levels in plasma and enhances $PGF_{2}\propto$ and PGE_{2} production in the pregnant rat which potentially increase the risk of preterm labour (Amira et al., 2010). Meanwhile, many studies showed that omega-6, AA contribute to increase mRNA and protein

expression of COX-2 which plays an important role as the coenzyme of prostaglandin type-2 synthesis and increases myometrial contractions as identified in human and animal studies (Allen and Harris, 2001; Kirkup et al., 2010; Novak et al., 2003; Roman et al., 2006)

Primary prostaglandins are formed from unesterified PUFA (AA or EPA). The free PUFA (AA, DGLA or EPA) is liberated through the activity of the phospholipase A2 (PLA2) enzyme. The phospholipid enzyme activates if it binds to oxytocin (Rice, 1995). Group IV cytosolic A2α (GIVA) is one group of phospholipases that play an important role in controlling the availability of free AA for Prostaglandin synthesis. The activation of cytosolic A2α is dependent on the release of calcium from the endoplasmic reticulum, which in turn leads to activation of PKC (Gijon and Leslie, 1999). The free AA or other PUFA are then converted to PGG or PGH that act as intermediates for formation of PGE and PGF. The PGH is then catalysed by Cyclooxygenase enzymes (COX) to produce Prostaglandin E receptor and Prostaglandin F receptor (Challis et al., 1997). The summary diagram of prostaglandin synthesis can be seen in **Figure 1.7.**

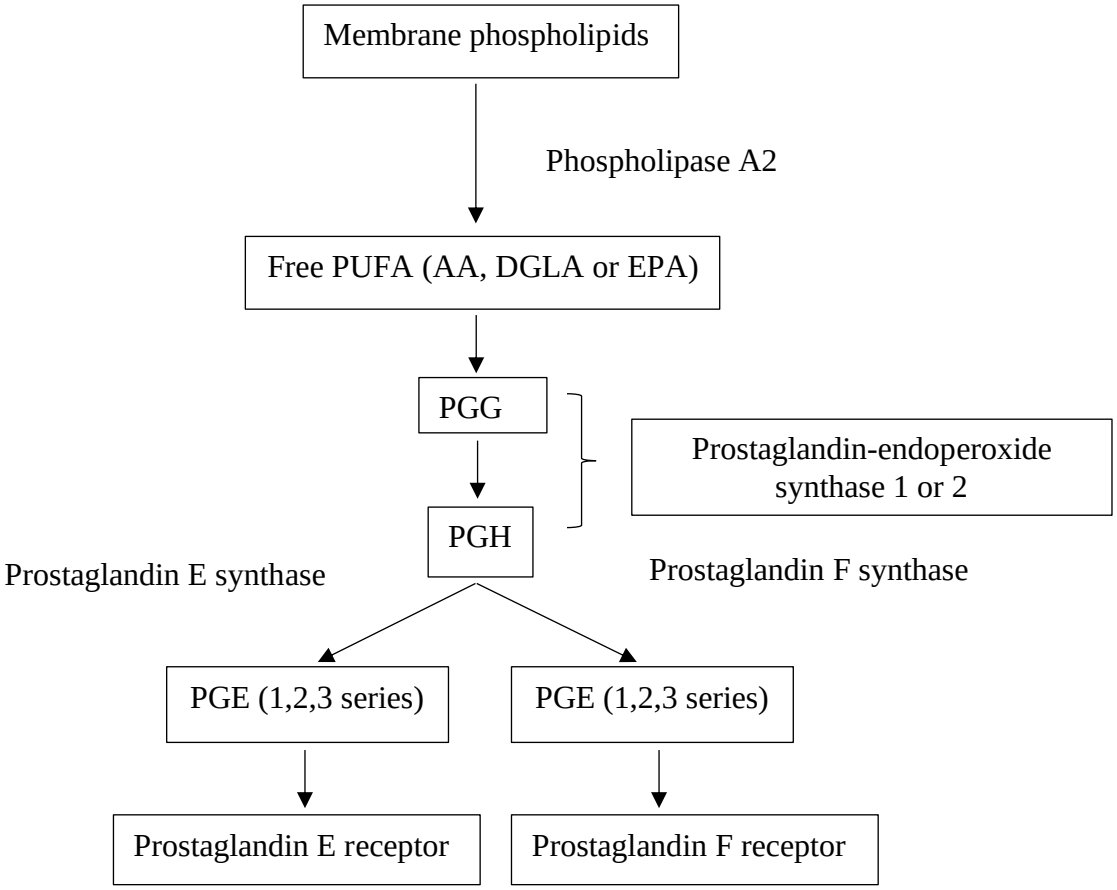

Figure 1.7 Summary diagram related to Prostaglandin Synthesis through PUFA metabolism. PGG : Prostaglandin G, PGH: Prostaglandin-H , AA : Arachidonic acid, DGLA : Dihommo Gamma Linoleic acid, EPA : Eicosapentaenoic acid (Wathes et al., 2007)

1.6.3.2 The role of fatty acid in regulating connexin signalling

Connexins are regulated by fatty acids through interaction with membrane receptor. Acute Fatty acid (FA) exposure, especially from medium and long chain polyunsaturated fatty acids (PUFAs), could interact and activate the membrane receptor or Free fatty acid receptor (FFARs) leading to enhance Connexin abundance or modify the gap junction channel (Ichimura et al., 2014). FFAR is G family coupled receptor which play an important role in signalling molecule pathways by interacting with protein kinase C (PKC), Mitogen-activated protein kinase (MAPK), or Protein Kinase B (PKB) or serine/threonine protein kinase in cytoplasm and modify the opening of Hemichannels (HCs) and Gap Junction Channels (GJC). The fact that fatty acids can modify gap junction suggests that fatty acids could

modify Connexin (Cxs) abundance in the cellular membrane by changing the amount and distribution of Cx into HCs or GJc (Puebla et al., 2017). Furthermore, Fatty acids could activate Cx directly by altering protein synthesis, or activating intercellular signal pathways which can modify Cx assembly due to changes in the protein degradation or synthesis rate (Puebla et al., 2017).

PUFAs are known to affect the timing of parturition by altering the expression, distribution and post-translation of protein associated gap junction or Cx. This theory is supported by Hayashi et al., (1997) that reported the inhibition of Gap junctions after 1 hour treatment with LA in a rat liver epithelial cell line. The cells response to long term treatment with LA did not recover after washing out the extracellular solution. Moreover, 1 hour treatment of epithelial cells with LA decreased CX-43 mRNA expression suggesting PUFAs are involved in the regulation of post translation modification. Furthermore, evidence showed lower protein expression of Cx-43 after exposure to a high fat and high cholesterol diet compared to control diet during pregnancy in the rat suggesting a potential mechanism behind prolonged labour with maternal obesity (Elmes et al., 2011).

However, another fatty acid, Oleic acid (OA) can both inhibit and stimulate myometrial contractions since a study conducted by Cheng et al., (2015) identified that maternal endometrium from sheep treated with OA increased production of PGE_2 but attenuated $PGF_2\alpha$ production leading to a doubling in ratio of PGE_2:$PGF_2\alpha$. Further evidence provided by Huang et al. (2004) is that OA induces CX-43 Ser368 phosphorylation which induces gap junction disassembly in rat cardiomyocytes. If OA increases phosphorylation of CX-43 in the myometrium, it could be a possible mechanism behind unsynchronised contractions, and Muir et al., (2016) did in fact report that a HFHC diet increased phosphorylation of CX-43 (PCX-43) that was associated with unsynchronised myometrial contractions in the term pregnant rat.

1.6.3.3 Effect of Fatty acids on Caveolae

There is evidence to suggest that omega-3 PUFA have the highest effect to increase membrane fluidity, therefore it potential to influence caveolae function and composition where many protein signalling abundance (Majkova et al., 2010). This theory supported by Li et al., (2007) that provides evidence that administration of the omega-3 EPA significantly increases eNOS activity in human umbilical vein endothelial cells. In addition, Immunofluorescence also confirms that omega-3 administration into human umbilical vein endhothelial cells induce Caveolin-1 and eNOS distributed from the plasma membrane to cytoplasm of cells. Therefore, omega-3 affects redistribution and translocation of Cav-1 from the plasma membrane to cytosol or the intracellular cell (Li et al., 2007). However, saturated fatty acid also could affects protein Cav-1 as study conducted by Elmes et al., (2011) provides further support that obesity induced by a high fat and high cholesterol diet also decreased protein expression of Cav-1 in the rat uterus during labour and could be a potential mechanism behind dysfunctional labour with maternal obesity.

From all the evidence above, a type of fat, especially saturated fatty acid potential to suppress contractions through decreased protein expression of Cav-1, while omega-3 could potentially increase contractions through translocation or change in the distribution of Cav-1 from the plasma membrane to the cytoplasm which may possibly be associated with other signalling molecules such as RhoA and RoK leading to increased Ca sensitization and myosin light chain phosphorylation (MLCK) to enhance contractility (Somlyo et al., 2003).

1.6.3.4 The effect of fatty acids on Peroxisome Proliferator Activated Receptors (PPARs)

Fatty acids, or their derivatives such as eicosanoids, can interact with nuclear receptor proteins such as PPAR. It has been reported that one eicosanoid derived from arachidonic

acid, prostaglandin J2 (PGJ2), binds with PPARγ which might be important in labour. The omega-3 PUFAs, such as ALA and DHA, and the omega-6 PUFAs, such as LA and AA, bind with certain PPARs. Moreover, saturated fatty acids, such as myristic acid and stearic acid, have been shown to bind with PPARα (Varga et al., 2011). Another study also proved that omega-3 PUFA increased PPARα activity in the liver, while gamma linoleic acid and EPA induced COX-2 protein synthesis via PPARγ in human immortalized keratinocytes cells (HaCaT) (Chene et al., 2007). The same mechanism may be true for omega-3 and omega-6 to alter PPAR expression in the placenta and affect myometrial contractions during labour.

Furthermore, SFAs and PUFAs perform opposite actions; SFA is mostly associated with metabolic pathways that are associated with steatosis like increased level of transcription factors (mRNA and protein) of SREB-1 and mediates hyperlipidaemic effects via gene peroxisome proliferator-activated receptor g coactivator-1b (PGC-1b) in the cultured rat liver (Lin et al,m 2005). Another study provides evidence that omega-3 EPA and DHA binding with PPARα decreases mRNA expression of IL-1β and TNF α suggesting that omega-3 can control inflammatory cytokines through PPARα (Zuniga et al., 2011). Many studies have reported that increasing protein expression of PPARα has the potential to enhance fatty acid oxidation and decrease lipid storage preventing lipid accumulation (Gorniak, 2013). One study reported that increasing triglyceride accumulation in the myometrial myocytes in pregnant women might play a role in the pathophysiological mechanism behind labour dystocia in obese women (Gam et al., 2017),

1.6.3.5 The effects of Oleic acid (OA) in smooth muscle contraction

Some studies have reported the effects of OA on prostaglandin production, PKC activation and on Gap Junctions, which may potentially alter myometrial contractions during parturition (Cheng et al., 2015; Diaz-Guerra et al., 1991; Hirschi et al., 1993; Khan et al.,

1991; Tunaru et al., 2012). One study established that oleate did not affect phorbol ester and Diacylglycerol (DAG) binding sites of the PKC enzymes, but oleate exposure increased systolic calcium-free concentration and activated Ca^{2+} dependent PKC isoenzymes through the stimulatory effect of DAG in rat isolated hepatocytes (Diaz-Guerra et al., 1991). The same study also provide evidence that OA can also promote a redistribution of protein expression of PKC from the cytosol to the plasma membrane but it requires Ca^{2+} for the translocation. OA causes a translocation of a conventional PKC such as PKC $\gamma$to the membrane but only shows little effect on PKCε (Pany et al., 2012)

OA has been reported to alter the outcome of labour by increasing the ratio between PGE_2: $PGF_{2\alpha}$ through increased NfkB and proinflammatory signalling pathway (Cheng et al., 2015). It has also been identified that castor oil rich in OA can activate the prostaglandin EP3 receptor of the intestine and uterine smooth muscle cells (Tunaru et al., 2012). A study conducted by Ishida et al. (2012) also supports the theory that OA increases the EP-3 receptor through activation of PKCδ pathways, leading to a stimulation in contractions of mesenteric arteries of the diabetic rat. The same mechanism of OA inducing the EP-3 receptor via PKC may potentially occur in the uterus and increase myometrial contractile activity during labour.

Furthermore, real time PCR conducted by Yasuda et al., (2007) revealed a significantly higher mRNA expression of PKCβ in myometrium preterm and term women compared to non-pregnant women. PKCβ potential to increase the phosphorylation of MLC and might increase actin-myosin activity that potential to increase contraction during parturition. The same study also provided evidence that PKCβ increases the amplitude of human myometrial contractions.

In contrast, Huang et al. (2004) provides evidence that OA induces Gap Junction disassembly through activation of PKCε, but PKCα inhibitors have no effect in preventing OA-induced disassembly of gap junctions in rat cardiomyocytes. The mechanism behind the

inhibition of contractions through PKCε, after OA administration could also occur in the uterus. Thus, OA might both stimulate or inhibit myometrium contraction depending on the type of isoform its activating.

1.7 The mechanism of normal labour

Progesterone is a key steroid for the maintenance of pregnancy but prior to the onset of labour, uterus activity changes from a quiescent to active contractile state. In late pregnancy, there is an increase of oestrogen synthesis that catalysed by aromatase in human placenta (Okubo et al., 1996). Increased oestrogen concentrations in turn lead to increased expression of the genes and receptors required for myometrial contraction such as prostaglandins and CAPs including COX-43, COX-2 and OXTR (Terzidou, 2007). Increased OXTR gene expression in uterine' smooth muscle cells induces contractions by increasing intracellular Ca^{2+} that activates MLCK in myometrial cells (Sanborns, 1998). In addition, the increased intracellular Ca^{2+} induces translocation of PKC β to the membrane increasing actin and myosin that inhibit MLC phosphatase, stimulating contraction in the myometrium after gestation (Ozaki et al., 2003).

Inflammation is now widely accepted as an important key of parturition; human labour is associated with the up-regulation of inflammation in the uterus and placenta (Christiaens et al., 2008). The review conducted by Baeuerle and Baltimore (1996) shows that NFkB triggers the transcription of many mediators including IL-1β, IL-6 and TNF-α in animal cells. Osman et al. (2006) provided evidence that macrophages are the major producers of these cytokines and that the decidua contains the largest concentration of leukocytes including neutrophiles and macrophages where the highest mRNA expression levels for the proinflammatory cytokines include IL-1β, IL-6, TNF-α and COX-2. Interestingly, cytokines also effect prostaglandins synthesis as Zaragoza et al. (2006) provided evidence that mRNA expression of prostaglandin F α receptor (PTGFR) increases when the human myometrium

ULTR cell line is incubated with IL-1β. The same study also showed that NfKb is involved in both basal- and IL-1β-stimulated transcriptions of the PTGFR gene. Furthermore, the study uncovered evidence that mice that lack IL-1 and TNF-α receptors have significantly lower myometrial expression levels of PGHS-2 mRNA.

$PGF_2\alpha$ has recently been shown to increase the protein expressions of MMP-2 and MMP-9 in human term decidua and the high expression levels of MMPs are potentially associated with membrane rupture (Ulug et al., 2001). A Review conducted by Christiaens et al., (2008) highlights that MMPs can increase proinflammatory cytokines causing a decrease in both the progesterone receptor and PTGFR in human and rodents study. The potential mechanisms regulating contractions during normal labour are presented in **Figure 1.8**.

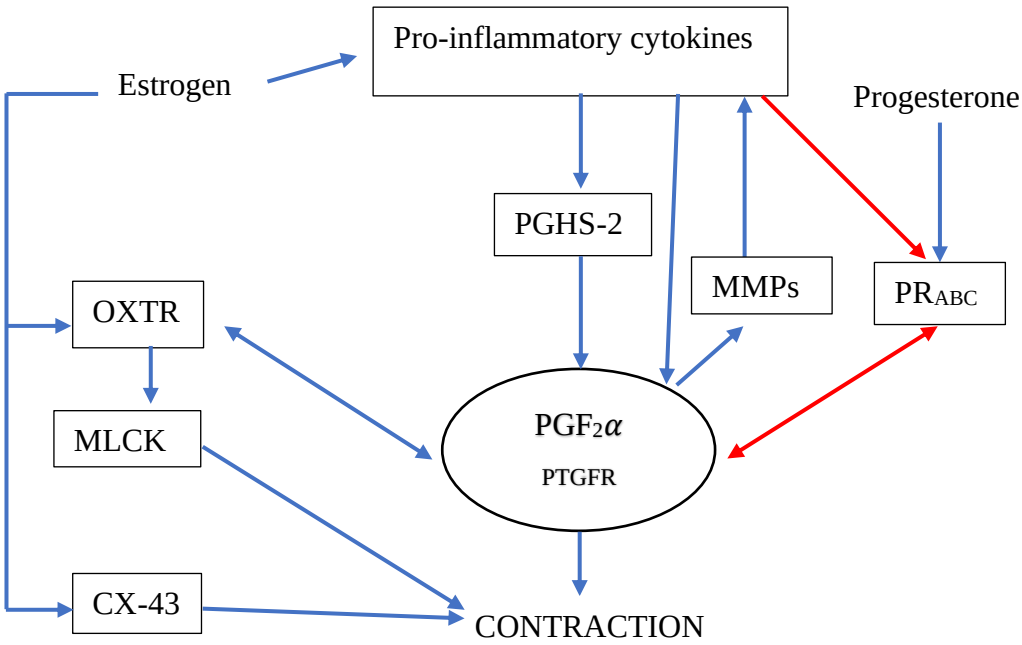

Figure 1.8 Mechanism of Normal Labour. Blue arrows represents stimulatory actions, while red arrows represent inhibitory actions. MMPs : matrix metalloprotein, $PGF_2\alpha$: Prostaglandin F2 α, PTGFR : prostaglandin F receptor, PR_{ABC} : progesterone receptor isoforms A,B,C, OXTR : oxytocin receptor, VEGF : Vascular endothelial growth factor. Figure adapted from Christiaens et al., (2008) with modification.

1.8 The potential mechanism behind prolonged and dysfunctional labour in maternal obesity

The biological mechanism underpinning dysfunctional labour in maternal obesity is yet to be determined. At the term the lower section of the uterus becomes relaxed, and the upper region goes through a transition to become highly contractile. Therefore, the myometrium in the upper uterus becomes sensitive to contractile signals from hormones, including oxytocin and prostaglandins (Thomson, 2013). However, Obese mothers have fewer oxytocin receptors, and these are less responsive to physiological and synthetic oxytocin, resulting in weaker and a decreased frequency of contractions which are more difficult to augment (Carlson et al., 2015; Leo et al., 2009).

The existing reports about a dependent change in oxytocin receptor are still contradictory. One study reports that there are fewer oxytocin receptors (OXTR) in the myometrial biopsies of obese women at term labour (Cook et al., 1999). However, some studies report no change of OXTR gene and protein expression in obese mothers in human and animal trials (Grotegut et al., 2013; Muir et al., 2016). Contradictory findings could result from the fact that the stage of labour, the location of the biopsy, and the timing of pregnancy affect OXTR expression (Garfield and Beier, 1989).

Furthermore, the other potential mechanism related to dysfunctional labour in maternal obesity is associated with higher lipid accumulation in the myometrium (Gam et al., 2017; Herrera and Ortega-Senovilla, 2010). This theory is supported by Mouzat et al. (2007) who identified that a lack of the Liver X Receptor (LXR) receptor which regulates cholesterol homeostasis, leads to decreased myometrial contractility in the mouse. Obesity in pregnancy is associated with disruption of the normal metabolic response and the increasing of a dyslipidaemia environment, indicated by the high level of plasma concentrations of triglycerides, cholesteryl esters and very low-density lipoprotein and low level of high-

density lipoprotein. Increasing triglyceride and cholesteryl esters is present in both obese human and animal myometrial strips which could potentially suppress myometrial contractile activity (Buxton and Vittori, 2005; Herrera and Ortega-Senovilla, 2010; Smith et al., 2005b). Mouzat et al. (2007) identified that the accumulation of cholesteryl esters caused uterine myocytes to be less responsive to a higher concentration of oxytocin and $PGF_{2\alpha}$ due to muscular defects. This could be a potential mechanism behind prolonged and dysfunctional labour associated with maternal obesity.

A study reported that a high level of cholesterol in small strips of non-pregnant and non-labouring human myometrium biopsies is associated with an increase in oxytocin-stimulated contractions, decreased uterine force and Intracellular Ca^{2+} *in vivo* (Jie et al., 2007). These findings are supported by Muir et al. (2016) who demonstrated that maternal obesity is associated with unsynchronised myometrial contractile activity in the pregnant rat. From the same study, it also reported that protein expression of CX-43 and OXTR decreases but progesterone production increases significantly at term labour but fell significantly to a similar concentration to the control group during labour. Increased progesterone production in the HFHC pregnant group at term of pregnancy suggests the possible mechanism is associated with oestrogen synthesis in HFHC group rat which results in unsynchronised myometrium contractions. Normally, progesterone production increase slightly while oestrogen production increase dramatically during parturition. The progesterone production will activate uterine NO synthetase, which is a major factor in uterine quiescence, and prevent tight regulation of prostaglandin production, calcium channels, oestrogen receptor and oxytocin receptor expression and interleukin synthase which are all associated with uterine relaxation (Vannucini et al., 2016). Furthermore, obesity in pregnancy is associated with prostaglandin insensitivity (Carlson et al., 2015). The study conducted by Konopka et al. (2013) mentioned that insensitivity of PGE_2, a two series prostaglandin can inhibit

myometrial contractions which may be associated with the failure of progesterone withdrawal in the human study.

Aromatase is a crucial enzyme that controls oestrogen synthesis, the hormone that increases dramatically at term and potentially increases myometrial contractions (Mueller et al., 2006). However, PPAR γ has the potential to inhibit aromatase in the placenta since a study conducted by Subbaramah et al., (2012) proves that activation of PPAR γ suppresses protein expression of aromatase through an elevated level of 15-PGDH, the enzyme that plays a major role in the catabolism of PGE2 in female mice mammary gland cells. The same mechanism possibly occurs in the placenta with maternal obesity since maternal obesity is associated with increased free fatty acid circulation that is associated with increased expression of both PPAR γ mRNA and protein in obese ewes at mid-gestation (Zhu et al., 2010). The inhibition of aromatase enzymes potentially leads to a decrease in oestrogen synthesis resulting in inhibition of myometrial contractions (Duttaroy, 2009).

In the placenta of obese women, there is a higher level of triglyceride accumulation compared to the placenta of women of a healthy weight that is caused by either an impaired or oversupply of fatty acids (Oakes et al., 2013). The higher lipid accumulation in the placenta of obese women might be caused by changes in placental fatty acid handling such as fatty acid uptake, a decrease of fatty acid oxidation, and an increase in esterification which increases the supply of lipid to the foetus and foetal adiposity. Moreover, a greater level of lipid storage is usually associated with placental inflammation in maternal obesity (Calabuig-Navarro et al., 2016).

There is some evidence to suggest that there is an association between PPARs expression including PPARγ and PPARα with proinflammatory cytokines (Froment et al., 2006; MacLaren et al., 2006; Pascual et al., 2005; Schaiff et al., 2006). The evidence that PPARγ plays a role in suppressing myometrial contraction might be related with inhibition

of inflammatory cytokine since many studies have revealed that low activation of PPAR γ in the placenta can increase pro-inflammatory molecules such as IL-β, TNFα, IL-6 and IL-8 and potentially stimulate contractions during labour (Borel et al., 2008). Furthermore, a study conducted by Dong et al., (2006) suggests the same mechanism where decreased mRNA and protein expression of PPARα was associated with labour while mRNA and protein expression of IL-1β increased significantly in human myometrium. This all suggests that high mRNA and protein expression of PPAR γ in the placenta and high mRNA and protein expression of PPARα in the uterus might be associated with pro-inflammatory inhibition and could be the mechanism behind dysfunctional labour with maternal obesity.

There is evidence to suggest that the amount of saturated fatty acid within the placenta of overweight and obese women is lower after omega-3 PUFA supplementation compared with controls (Calabuig-Navarro et al., 2016). The omega-3 PUFAs regulate the expression of key genes involved in lipid metabolism and energy utilisation such as fatty acid synthesis, oxidation and decrease lipid storage (Khaire et al., 2015). A recently published study reported that a high fat and high cholesterol diet increases saturated fatty acid and omega-9, and decreased omega-3 and omega-6 in the plasma and liver, but only omega-3 decreased significantly in the uterus of the rat (Muir et al., 2018). Interestingly, the effect is reversed when the diet changes from high fat, high cholesterol to a control diet during pregnancy. Thus, suggesting that polyunsaturated fatty acids, especially omega 3, PUFAs have the potential to alter labour outcomes with maternal obesity.

Fatty acids could both directly alters CAPs such as protein CX-43 and Cav-1 in the membrane and affect the assembly of gap junction and other proteins that mediate smooth muscle contractility. Moreover, fatty acids could translocate from the membrane to the cytoplasm through interaction with CD36 and FABP that facilitate the entry of fatty acid to the cell which then triggers muscle contraction through the activation of PKC, binding with

PPAR, mediate prostaglandin type-2 synthesis and pro inflammatory cytokine (Glatz and Luiken, 2018; Guttler et al., 2014; Poebla et al., 2017). This theory is supported by the fact that omega-3 consumption prolongs gestation and labour in mice and sheep (Baguma-Nibasheka et al., 1999; Harper et al., 2010; Yamashita et al., 2013). In contrast, Omega-6 especially AA has been reported to do the opposite and has the potential to increase myometrial contractions through enhanced protein expression of COX-2 as a coenzyme for prostaglandin type 2 synthesis in animal studies (Cheng et al., 2013; Schuler et al., 2006). Other studies proved that consumption of a high LA diet in late pregnancy enhance maternal and foetal PGFM and foetal PGE_2, also maternal oestradiol concentrations which can increase the risk of premature labour in the ewes (Elmes et al., 2004). Meanwhile, OA has been shown to decrease amplitude and frequency of myometrial activity of contraction in pregnant uterus strips rat under oxytocin stimulation (Hag et al., 2019). Another study conducted by Kim et al., (2003) proved the evidence that PKCα express in rat myometrium and exposure of PKCα activator inhibits the tension induced by high K+ in the uterus.

1.9 Aims and Hypotheses

Expanding further findings on the aetiology and mechanism behind maternal obesity associated dystocia is an important step in improving the labour outcome for the obese parturient.

The overall purpose of the present study was to investigate the potential mechanism behind prolonged and dysfunctional labour associated with maternal obesity. is The hypothesis to be tested is that dysfunctional myometrial contractile activity is caused by diet rather than the physical effects of obesity.

To test this hypothesis the study aims :

1. To identify the effects of exposure to a HFHC diet during pregnancy on lipid levels in the uterus (Chapter 2).
2. To investigate the effect of dietary HFHC induced maternal obesity and change of diet from HFHC diet to control and vice versa at conception on fatty acid composition (%) of the plasma, liver and uterus (Chapter 2).
3. To identify whether changing diet from HFHC diet to control and vice versa at conception causes changes in fatty acid composition that correlate with plasma prostaglandin type-2 concentrations (Chapter 2).
4. To determine whether the HFHC diet may impact on myometrial contraction through placental protein expressions involved in steroid production such as PPARs and aromatase (Chapter 3).
5. To identify the effect of different ratio of dietary omega-3 (Alpha Linolenic acid) and omega-6 (Linoleic acid) fed at different fat levels at conception on on uterine expression of contractile associated proteins such as CX-43, CAV-1, COX-2, OXTR, PCX-43 (Chapter 4).
6. To investigate the effect of direct exposure to OA on myometrial contractile activity *ex-vivo* in the organ bath study (Chapter 5).

2. The Effect of Exposure to High-Fat and High-Cholesterol Diet during Pregnancy on Uterine Histology, Fatty Acid Composition and prostaglandin production in Pregnant Rats

2.1 Introduction

The prevalence of obesity among pregnant women varies depending on the population, and ranges from 8 % to 30% where prevalence of obesity among UK pregnant women is 26% around 2002 to 2004 (Guelinckx et al., 2008; Shaikh et al., 2010). Between 1990 and the year 2000 maternal obesity increased 7-10% in England (Heslehurst et al., 2010). In England it has been identified that more than 50% of women of reproductive age (25-44 years old), are currently obese (HSCIC, 2016). Moreover, Bogaerts et al. (2013) reported that maternal obesity is associated with many complications during pregnancy, including miscarriage, preeclampsia, gestational diabetes, postpartum haemorrhage, labour induction and prolonged and dysfunctional labour. The relationship between maternal obesity and inefficient uterine contractility is still unresolved. Therefore, identifying the mechanism behind dysfunctional labour in obese pregnancy could be a potential strategy for intervention.

A recent study reported that High fat and high cholesterol feeding increases cholesterol and triglyceride concentration in both the plasma and myometrial tissue with the potential to inhibit spontaneous contractions in myometrial strips in both humans and animals (Elmes et al., 2011; Gam et al., 2017; Smith et al., 2005b). During the last trimester of pregnancy, accumulation of maternal fat stops as a result of increased adipose tissue lipolytic activity. In contrast, insulin resistance associated with maternal obesity has been shown to contribute to reduced insulin suppression of lipolysis that could potentially increase fat accumulation (Jarvie et al., 2010). Herrera and Ortega-Senovilla (2010) explained that the significantly high level of plasma insulin combined with insulin resistance in obese women has been shown to contribute to decreased

lipolysis in the adipose tissue, and stimulates free fatty acid uptake and fat storage in skeletal tissues. A study conducted by Scrauwen_Hinderling et al., (2016) supports this theory and reported that elevated plasma FFA levels or diet high in fat are known to increase intramyocellular lipid content in skeletal muscle tissue, suggesting that skeletal muscle stores fat if the availability of fatty acids are high. This condition could potentially occur in the myometrium and be the mechanism behind prolonged and dysfunctional labour associated with maternal obesity.

The other potential mechanism of prolonged labour in maternal obesity is associated with prostaglandin type 2 synthesis since many studies reported that decreased production of prostaglandin type-2 hormones will reduce myometrial contractility at term labour (Challis et al., 2002a; Olson, 2003). Type 2 prostaglandins include $PGF_{2\alpha}$, PGE_2 and PGI_2 all of which alter myometrial contractions through different mechanisms. $PGF_{2\alpha}$ increases uterine activation proteins such as CX-43 and COX-2 in the upper human myometrium and increases oxytocin protein expression in the lower human myometrium. Indicating a role of $PGF_{2\alpha}$ in myometrial contractions at the onset of labour (Xu et al., 2013). Further studies identified that PGE_2 can maintain both uterine quiescence and stimulate contractions of the myometrium depending on its receptors where EP 1 and EP 3 stimulate contraction, while EP 2 and EP 4 maintain myometrial relaxation during pregnancy (Arulkumaran et al., 2012; Fetalvero et al., 2008).

Many animal and cell culture studies have reported that the fatty acids EPA, DHA, AA and OA are associated with alteration in synthesis of prostaglandins, PGE_2 and $PGF_{2\alpha}$. (Akerele and Cheema, 2016; Baguma-Nibasheka et al., 1999; Cheng et al., 2015; Cheng et al., 2013; Elmes et al., 2005). It has also been reported that GLA and AA exposure increase PGE_2 and $PGF_{2\alpha}$ and ratio of PGE_2 and $PGF_{2\alpha}$ until two fold in endometrial cells ewes in the late of gestation (Cheng et al., 2005). A study provide

evidence that Linoleic acid (LA) supplementation increases type 2 prostaglandin (PGE_2 and $PGF_{2\alpha}$), in endometrial ewes and increases the risk of preterm birth in pregnant ewes (Elmes et al., 2005). Meanwhile, omega-3 exposure suppresses the essential gene-regulating $PGF_{2\alpha}$ biosynthesis in the bovine uterus (Coyne et al., 2008). Increased EPA and DHA in the diet increase the $PGF_{2\alpha}$ -metabolite (PGFM) concentrations in cow plasma during 60 hours immediately after parturition (Mottos et al., 2004). Another study reported that decreased production of PGE_2 and $PGF_{2\alpha}$ in cultured decidual cells that had been preincubated with DHA and EPA, and that these fatty acids might inhibit myometrial contraction and decrease the incidence of preterm birth (Roman et al., 2006). One study further reported that the omega-6 PUFAs compete with the omega-3 and OA omega-9 PUFA to use desaturase and elongation enzymes for prostaglandin synthesis (Abayasekara and Wathes, 1999). Therefore, the different composition of LA, AA, EPA, DHA and OA within the uterus might be associated with plasma prostaglandin type-2 production and might contribute to adverse pregnancy outcome associated with maternal obesity.

There is evidence to suggest that a high fat and high cholesterol diet (HFHC) is associated with lower plasma $PGF_{2\alpha}$ expression compared with a control diet highlighting that maternal obesity is associated with low $PGF_{2\alpha}$ synthesis which may increase the risk of prolonged labour in the rat (Elmes et al., 2011) . This study supported by Muir et al. (2016) identified that chronic exposure to a HFHC diet decreases plasma and liver omega-6 and omega-3 PUFA composition and could be a contributing factor to the unsynchronised myometrial contractions at term labour in the maternally obese rat.

2.1.1 Objective and Hypothesis

The aims of the study was to investigate the effect of a HFHC diet on the number of vacuoles and mean vacuole area as an indicator of lipid accumulation in the uterus at term and during delivery (term non-labour and abour). This study also assessed the effect of a HFHC diet on the fatty acid composition of the plasma, liver, and uterus , and also to determine whether changing diet at conception from a HFHC to control diet during pregnancy and vice versa reverses the fatty acid composition . A further aim was to investigate whether there was a correlation between plasma prostaglandins with of the fatty acids DHA, AA, and EPA within the uterus. The hypotheses to be tested was that the uterus from rats fed a HFHC diet labour had greater vacuoles that indirectly represent a greater lipid incorporation within the myometrium and that a HFHC diet decreases incorporation of the precursor fatty acids for prostaglandin synthesis and have a negative correlation with plasma type 2 prostaglandin concentrations.

2.2 Materials and Methods

2.2.1 Animal Study

All animal experiments were performed under The University of Nottingham Guidelines and approved by The University of Nottingham Animal Welfare Ethical Review. The animal trial methods can be referred to Muir et al., (2018). A total of 40 virgin female Wistar rats (*Rattus rattus*) weighing approximately 60g were used for the study. All rats were pair-housed under 12 hours light; dark photoperiod and 21°C±5°C room temperature, and 55% relative humidity. Food and water were given by *ad libitum* and randomly fed either control chow diet (CON, n=20) and high fat and high cholesterol diet (HFHC,n=20). The breakdown of the ingredients to make HFHC diet is referred to in Elmes et al., (2011) as shown in **Table 3.1**, while the nutritional value of both the control

and HFHC diet is shown in **Table 3.2**. Rats were maintained on their respective diets prior to pregnancy for six weeks, then mated with Wistar male rats. The pregnancy was confirmed by the discovery of a semen plug later recorded as gestational day 0. Once conception had been confirmed, half the rats were maintained on their respective diet throughout pregnancy; control (n=20), HFHC diet (n=20 rats) while the other half switched diets throughout pregnancy, such that control fed rats switched to a HFHC diet (CON-HFHC=6) whereas HFHC feds rat switched to-Control diet (HFHC-CON=5). The rats were euthanised humanely through CO2 asphyxia and cervical dislocation did at two-time points; Term non-labour at gestational day 21 (TNL=10) and day 22 term labour (TL=10) following delivery of the first pup. At 22- days of gestation, rats were checked for the sign of parturition. A total number of 9 animals were not used in the trial as a result of congenital pathology or a lack of pregnancy. The maternal blood sample was collected via cardiac puncture into EDTA coated tubes (Sarstedt, Germany). Plasma was then separated using centrifugation at 13,000 rpm at 4° minutes. The uterus and placenta were dissected out, weighed and snap-frozen with liquid nitrogen then stored in -80°C. A section of uterus was also collected from rat dams that was fixed in formalin and stored in 70% ethanol for crude histological analysis.

Constituents	Amount (g/1010gr)
Corn oil	100
Casein	200
Maize starch	218
Butter	295
Sucrose	100
Cellulose	50
Vitamin Mix	5
Mineral mix	20
Methionine	10
Choline	2
**Cholesterol	10

Table 2.1 Breakdown of Ingredients used to produce HFHC Diet (**Important to note cholesterol was dissolved within the 100 ml vegetable oil)

Nutritional Value	Control	HFHC
The energy density (Kcal/g)	3.1	5.6
Total available carbohydrates (%)	58.9	30.5
Total Protein (%)	18.6	18.5
Total fat (%)	6.2	35.6
Total fibre (%)	3.5	4.8
Total ash (%)	5.3	1.9
Total moisture	7.5	8.8

Table 2.2 Nutrition Value of The Control and HFHC diet. Nutritional analysis was carried by Scientific Laboratory Services (SAIL) Ltd. U.K.

2.2.2 Histological analysis of the uterus

The histology analysis was used for investigating whether there is more lipid accumulation in HFHC uterus rat tissue compared with control tissue. The histology slide preparation consists of 4 crucial steps: fixing tissue, embedding tissue sectioning the specimen and staining the slides contain tissue. The author and Dambire Charlene, (South laboratory technician) also carefully handled and optimised the time of tissue embedding on tissue processor, so that the tissue was not damaged prior to sectioning with the microtome.

The rat uterine tissue from the four different treatment groups; high fat, high cholesterol and term labour (HFHC TL n=3), high fat high cholesterol and term non-labour group (HFHC TNL n=3), control and labour group (Control TL n=3), control and term non-labour group (Control TNL n=3) were used for histological analysis within eosin and haematoxylin staining to investigate the effect of HFHC diet on lipid deposits in uterine tissue. All tissues were fixed in 10% formalin then stored in 70% ethanol to preserve and maintain 'life-like' structure of tissue.

After fixation steps, the tissue samples were then cut into 1 cm cubes and placed into a tissue processing cassette. The tissue processor automated dehydration, clearing and infiltration paraffin wax within the tissue. The total time of tissue processor was 18 hours and the detail of solution and time for dehydration, clearing and infiltration is shown in Table 2.3. During the dehydration step, tissues are immersed into increasing concentrations of alcohol (70%, 80% and 95%) to remove water and formalin. Next, tissues were cleared with an organic solvent to remove alcohol and allow infiltration and infusion of the tissue with molten paraffin wax.

Process in Bath	Time (Hours)
70% ethanol	2
80% ethanol	2
95% ethanol	2
Absolute alcohol	2
Alcohol/histo-clear (v/V)	2
Histo-Clear	2
Histo-Clear	2
Parrafin	2
Parrafin	2

Table 2.3 Detailed time of tissue dehydration, clearing and Infiltration in the tissue processor

After 18 hours in the tissue processor, cassettes containing tissue samples were removed from the processor, and held at room temperature until required. Each cassette containing tissue samples were next filled with paraffin wax from the wax dispenser until it reached the top of the cassettes making sure all the tissue was covered. The samples were then removed from the cassettes with forceps and placed in the mould. The next step was to take the cassette base on the top of mould and then fill with more wax until the paraffin blocked the specimen. After that, the mould and cassette were placed in a cold place, approximately 4ºC for 30 minutes, until the specimen was ready for tissue sectioning using a rotary microtome. The tissues were cut 3-10µM thick using a rotary microtome (Leica 2125). Tissue sections were then allowed to float in a warm bath at approximately 37°C to flatten then the tissue section was carefully picked up and placed onto a glass microscope slide. After that, the microscope slide containing the tissue section was dried at 37°C to ensure the tissue adheres to the slide. Once sections had dried the slides underwent staining with haematoxylin and eosin. The protocol of Histology analysis is shown in **Figure 2.1.**

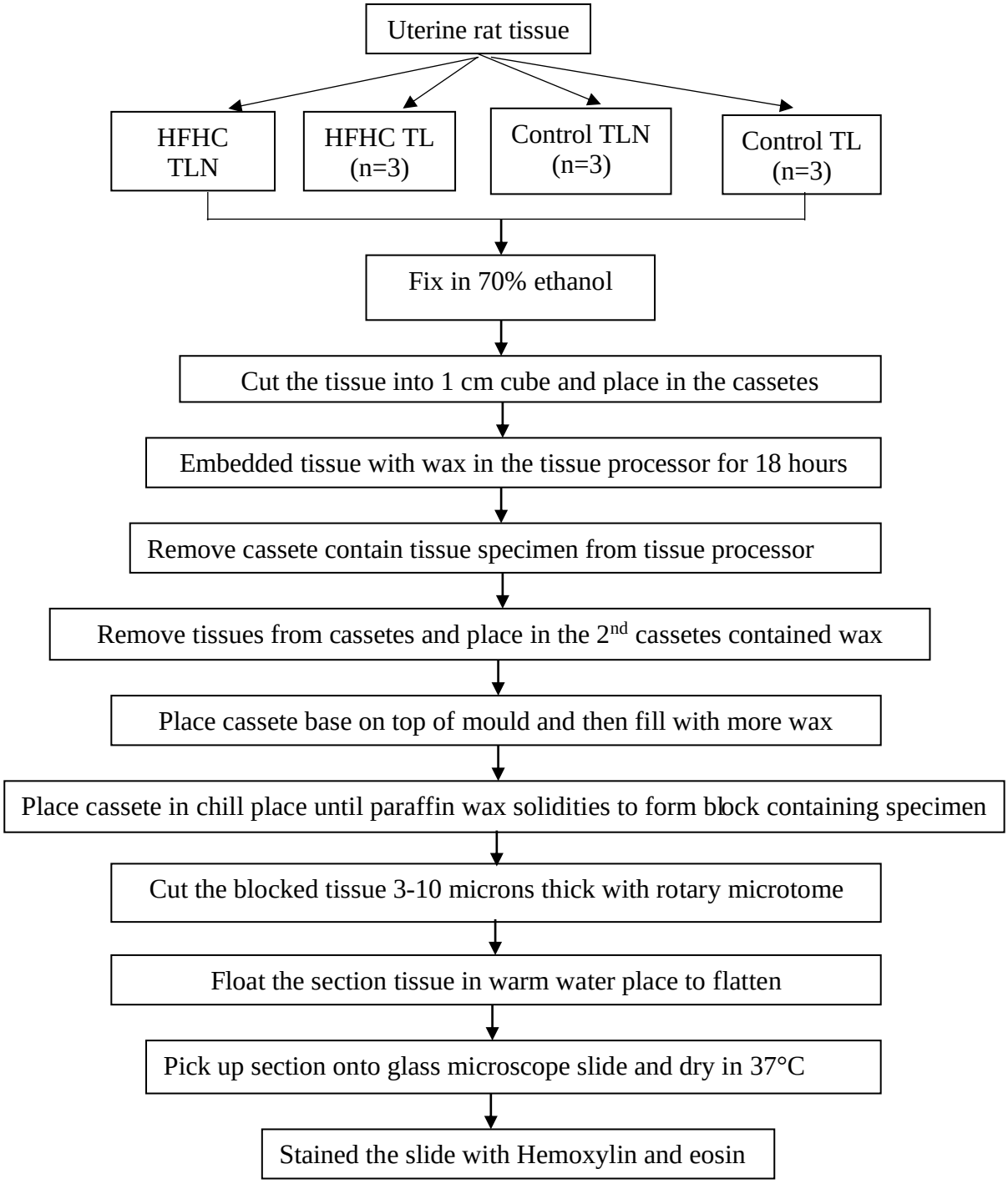

Figure 2.1 Histology Slide Preparation

The haematoxylin and Eosin staining used to colour different tissue structure. The haematoxylin has deep blue-purple colour and stains nucleus cell, while eosin has pink colour and stains cytoplasm and extracellular matrix. Before Haemoxylin and eosin staining, the slides were rehydrated by immersion in Histo-Clear solution (Fisher

Scientific) for 10 minutes. After that, the slide was immersed again for a 2nd time in Histo-clear solution (Fisher Scientific) for 10 minutes. The slides were then placed into decreasing ethanol concentration solutions (100%, 95% and 50%) in each solution for 2 minutes. The slides were then placed into water for 2 minutes before staining with H&E solution. The staining step began by Immersing the slides in Harris haematoxylin solution (Merck Sigma-Aldrich) for 15 minutes. After that, the slide was rinsed under tap water for 2 minutes. The slide was then dehydrated 2-5 dips in 70% ethanol containing 0.1% HCL for 1 minutes. Once complete, slides were washed for 2 minutes under tap water before being stained with eosin. Slides were immersed in 1% Eosin containing 0.1 acetic acid (Merck Sigma-Aldrich) for 10 minutes. Upon completion each slide was then immersed in the tap water again for 2 minutes. After the staining process, the slide was dehydrated and cleared in using different ethanol solution concentrations (50%, 95%,10%) for 2 minutes. Following dehydration, the slide was placed in Histo-Clear solution for 10 minutes twice. The last step was mounted the coverslip using Histomount (Scientific Laboratory supply). The procedure of haematoxylin and eosin staining is presented in **Figure 2.2**. The vacuoles were observed with 20X magnification of a microscope, and the number and mean area of vacuoles were calculated with Image J software.

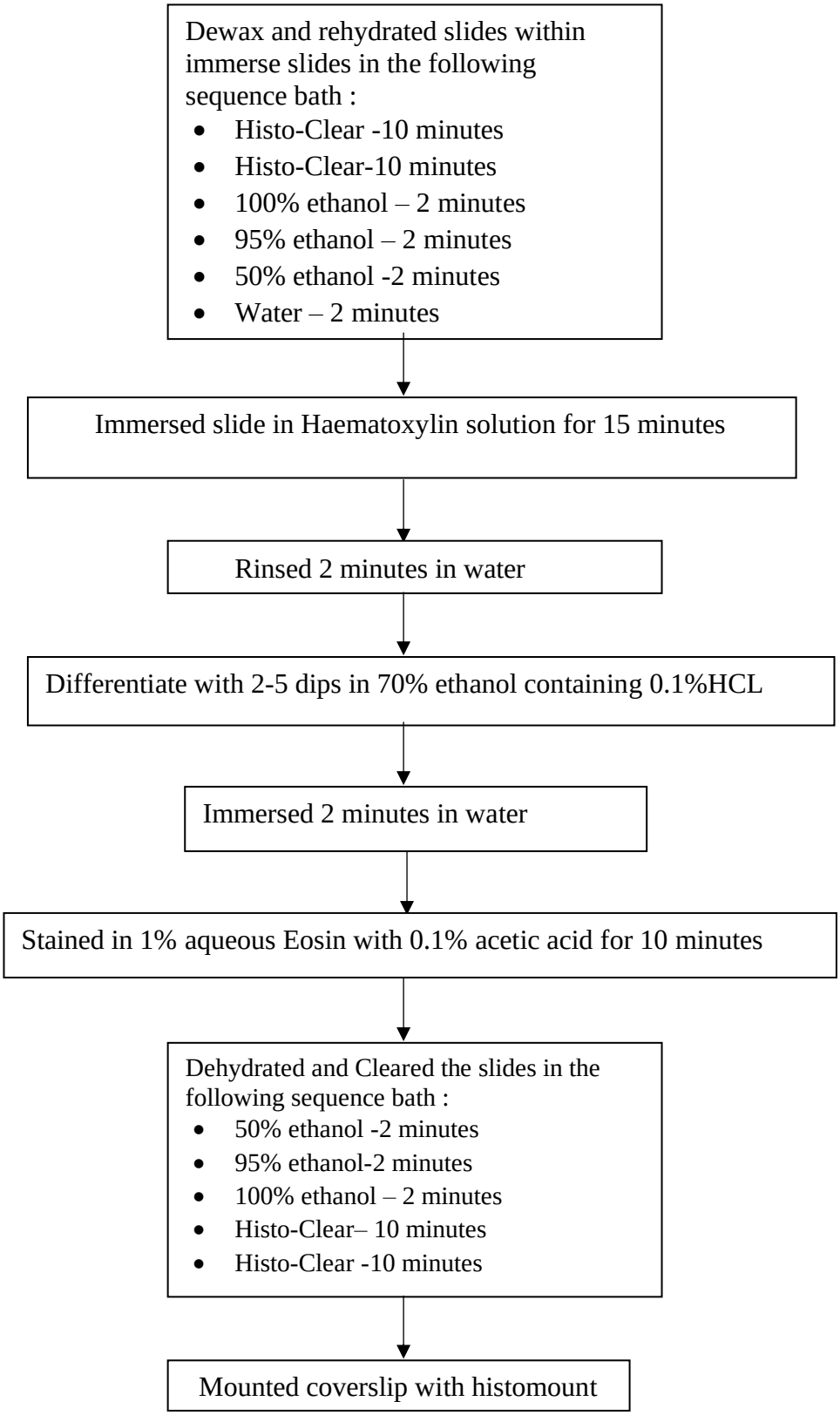

Figure 2.2 The Procedure of Haematoxylin and Eosin Staining

2.2.3 Lipid extraction and fatty acid analysis

2.2.3.1 Lipid extraction in the liver and uterus

Frozen rat liver and uterine samples 300 mg in weight were placed into a flat bottom glass laboratory tube. The uterine horn and liver samples were then manually homogenised in 2 ml cold 0.9% saline in 30-second bursts. The homogenated sample was then vortexed in 3 ml propan-2-ol, and allowed to incubate for 5 minutes. After incubation, 6 ml of chloroform was added to the solution, and the cap put on. Samples were shaken well and allowed to incubate for a further 5 minutes. Samples were then centrifuged for 10 minutes at 3000rpm (1559 xg). Following centrifugation, the chloroform layer was transferred using a long pipette into a glass test tube and dried under nitrogen gas at 37°C. Once evaporated, liver samples were reconstituted in 500 µl 9:1 chloroform: methanol, while uterine samples were reconstituted in 100µl chloroform: methanol. Then 20 µl of the solution was pipetted on PUFA coat collection card as described in Liu et al. (2014) for fatty acid analysis.

The plasma sample approximately 20 µl pipetted on PUFA coat collection card for fatty acid analysis as the same method as liver and uterus fatty acid analysis. The fatty acid analysis was conducted at the University of Adelaide.

2.2.3.2 Fatty acids analysis

The fatty acid profile of plasma, liver and uterus were analysed by Dr Ge Liu and Dr John Carragher of the Waites Lipid Analysis Service at the University of Adelaide. Samples were trans-esterified with 2 ml of 1% sulphuric acid in anhydrous methanol, and the fatty acid methyl esters (FAMEs) were extracted with heptane. The FAMEs were then separated, and analysed by Hewlet-Packard 6890 GC equipped with a capillary column (30mx0.25 mm) coated with 70% cyanopropyl polyphenylene-siloxane (BPX-70; 0.25µm film thickness) which was fitted with flame ionisation detector. The injector temperature was set at 250°C and FID temperature at 300°C, a

programmed temperature ramp (140-240°C) was used and Helium gas was utilised as a carrier at flow rate 35 cm/seconds in the column and the inlet split ratio was set at 20:1. The identification and quantification of FAME were achived by comparing retention time with peak area values of samples to the commercial lipid standard (NU-Chek Prep Inc., Elysian, MN, USA) using the Hawlett_Packards Chemstation data system. The explanation of principle Gas Chromatography FID is shown in **Figure 2.3**.

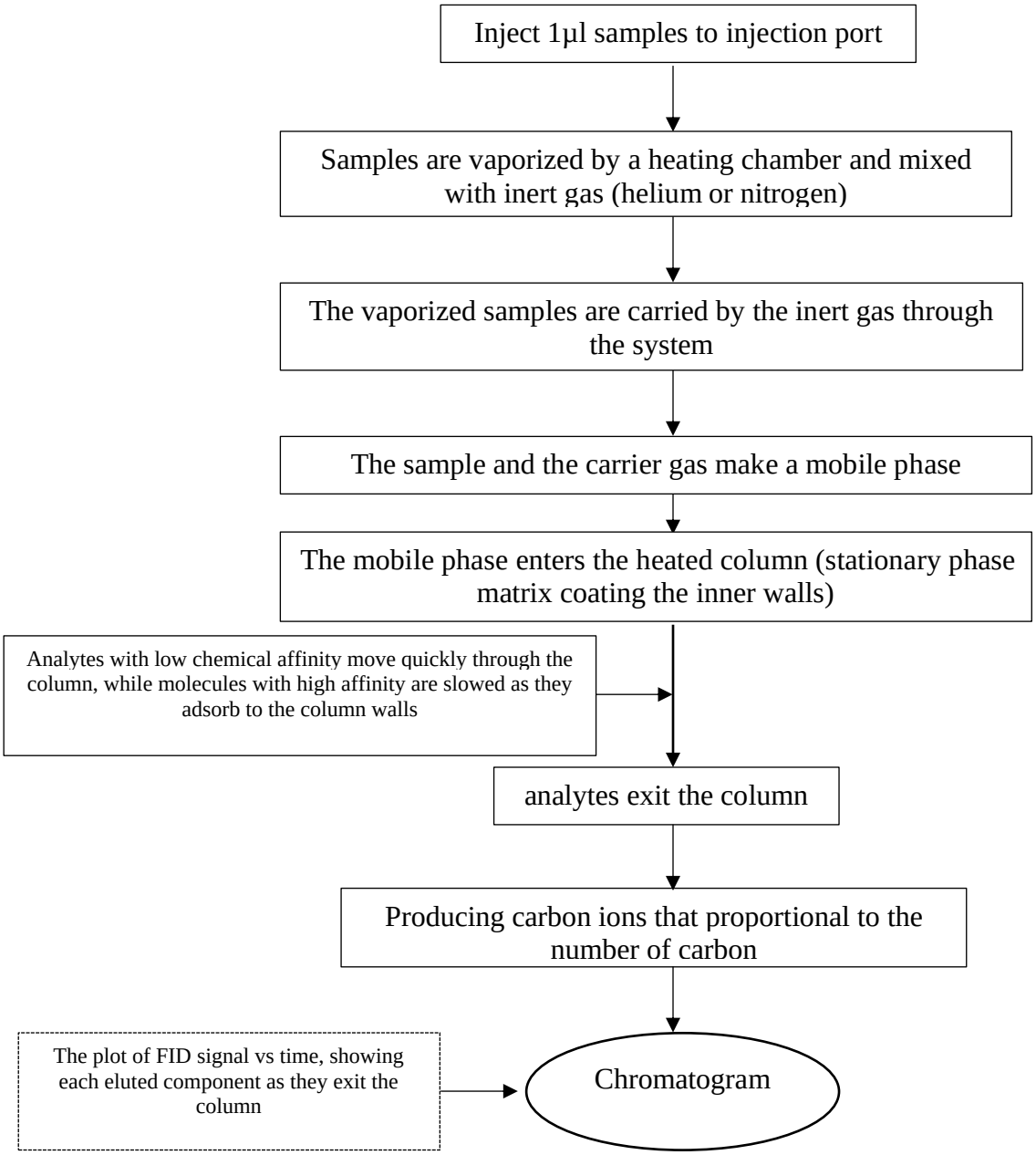

Figure 2.3 Principles analysis of Gas Chromatography FID (Venton, 2017)

2.2.4. Prostaglandin type-2 analysis

2.2.4.1 The plasma $PGF_{2\alpha}$

The plasma $PGF_{2\alpha}$ analysis was carried out with an ELISA kit bought from Abcam, with kit name $PGF_{2\alpha}$ high sensitivity ELISA kit (ab133056), following the kit instructions. A number of reagent and samples were allowed to equilibrate at room temperature prior to use, including 1X wash buffer and prostaglandin synthase inhibitor. The 1X Wash buffer was made by diluting 5 ml of the 20X wash buffer in 95 mL of ionised water then mixed gently but thoroughly, while 10 µg/mL prostaglandin synthase inhibitor was prepared by diluting 250µg ibuprofen into 500µL of 70% ethanol which then was added into plasma samples. After that, the standard solutions were made with concentrations of; 2500, 1250, 625, 313, 156, 78.1,and 39.1(pg/mL).

Assay buffer approximately 100 µL was added into Non-Specific Binding (NSB) wells. Then, 100µl of Assay buffer was added into Bo (0pg/mL standard) wells. The 100 µl standard reagent was added then followed with 100 µL of 20X diluted sample (5 µl sample in 95 µl assay buffer) were pipetted into appropriate wells. After that, 25 µL of $PGF_{2\alpha}$ conjugate blue reagent was added to Bo and NSB wells. The next step was a 25 µL PGF_2 antibody was pipetted into Bo, standard and samples wells. Every well used should be green in colour except the NSB wells which should be blue. The (Blank) Bs and (Total activity wells that only contained conjugate and substrate) TA wells which are empty at this point and have no colour. The plate then was covered with the plate sealer provided and incubated on a plate shaker at 500rpm for 5 minutes continued an overnight incubation at 4°C. During the second incubation, the antigen was bounded with specific antibody and conjugate. After incubation, the

contents of all the wells were emptied and washed with 400 µl of 1X washing buffer. This wash step was repeated twice. After the final wash, the wells were aspirated and the plate tapped on lint-free paper towel to remove any remaining wash buffer. Then, 2.5 µL of PGF_2 -A.P. conjugate was pipetted into T.A. wells (Total activity; contains conjugate and substrate). Then, 200 µL of pNpp Substrate solution was added to every well, and incubated at 37°C for 45 minutes for 3 hours without shaking for $PGF_{2\alpha}$ analysis. When the substrate was added, the samples with high antigen concentration generated more signal than those with low antigen concentration, producing a signal directly proportional to the number of antigen samples. The product was coloured by Pnpp substrate gave the signal that the ELISA kit works and need to stop. 50µL of stop solution was pipetted into each well to stop the reaction. The plate was then read on O.D. absorbance at 405nm.

2.2.4.2 The plasma PGE_2

The plasma PGE_2 ELISA kit was bought from Abcam, with kit code ab133021. All materials were same as the $PGF_{2\alpha}$ kit as mentioned in Section 2.2.4.1, and standards solutions were made with the same protocol and concentrations as mentioned in section 2.2.4.1. Before starting the assay, all materials and reagents were prepared at room temperature prior to use.

The assay was started by adding 100 µL of assay buffer into Non-Specific Binding (NSB) wells and Bo (0pg/mL standard) wells. Next 100 µL of prepared standards and 100 µL of diluted samples were added. Each unknown sample was diluted by a factor of 20X (5µl sample in 95 µl assay buffer). Then, 50 µL of assay buffer was pipetted into the NSB wells only. Every well used should be green except the NSB wells which should be blue and Bs ad TA which should be no colour. After that, the plate was covered with a sealer,and then incubated at room temperature on a

500 rpm plate shaker for 2 hours. After incubation, the contents of the wells were emptied and the plate was washed by adding 400 µL 1X wash buffer. This wash step was repeat 3 times and after the final wash, the wells were then aspirated and the plate firmly tapped on a lint free paper towel to remove any remaining wash buffer. Next 5 µL PGE_2 Alkaline phosphatase conjugate was added to the TA wells, followed by 200 µL of pNpp substrate solution to all wells. The plate was then incubated at room temperature for 45 minutes. After 45 minutes incubation, 50 µL stop solution was added to all wells and the plate was read immediately by a spectrophotometer with optical density set at 405nm. The principle of $PGF_{2\alpha}$ and PGE_2 analysis using ELISA assay is presented in Figure 2.4.

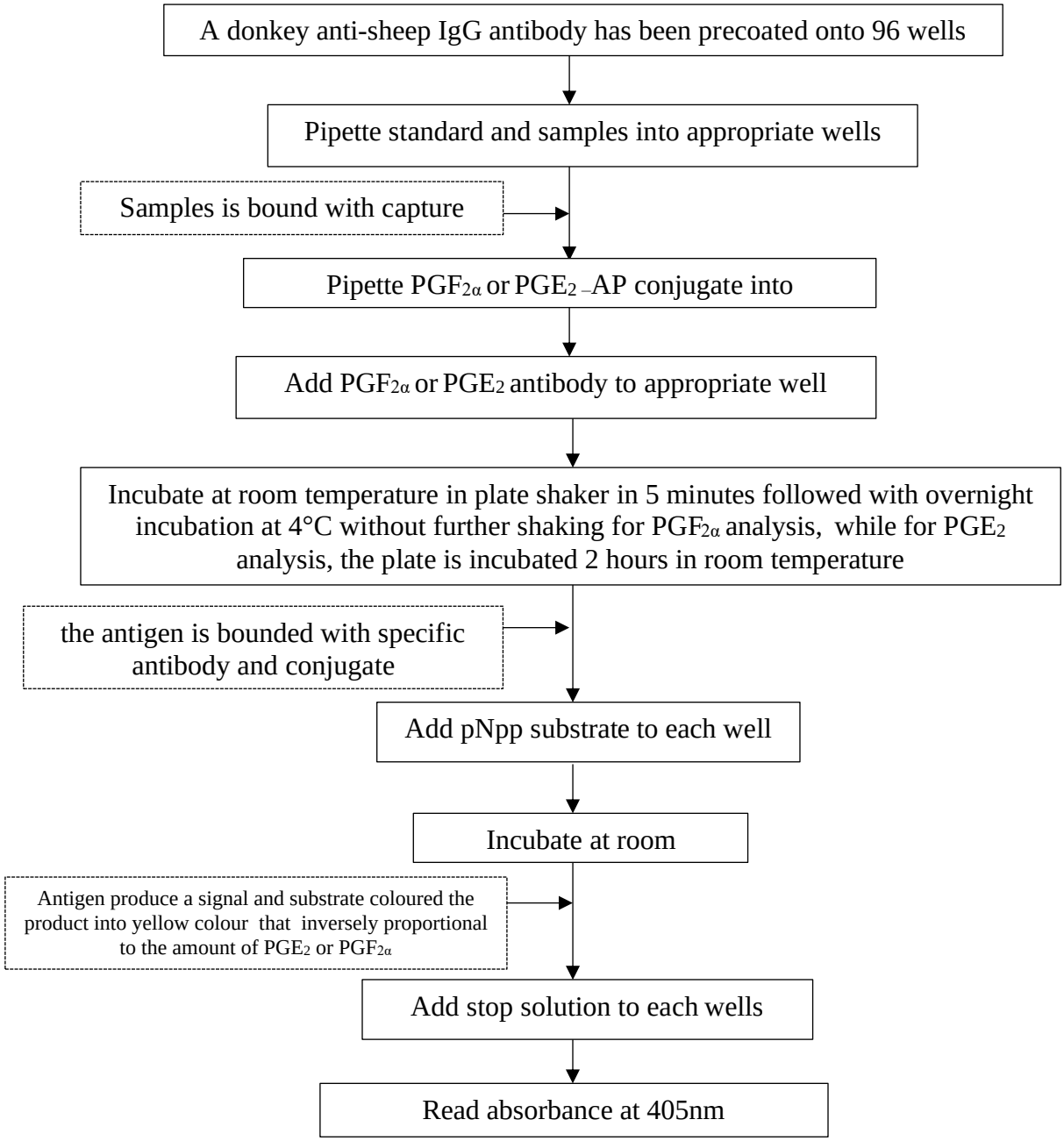

Figure 2.4 The process of $PGF_{2\alpha}$ and PGE_2 assay with ELISA kit

2.2.5 Statistic analysis

All statistical analysis was carried out using IBM SPSS version 24 software. The homogeneity of the data was assessed, if the data was not normally distributed it was appropriately transformed with log 10. If homogeneity could not be achieved a non-parametric Friedman test was used. The effect of the HFHC and control diet at term

or during labour on the number of vacuoles and vacuole mean area within uterus sections, were determined by two way ANOVA, while one way ANOVA was used to assess the effect of changing diet at conception on the plasma, liver and uterine fatty acid composition. If the ANOVA test revealed significant differences, the LSD post hoc test was used to determine the differences between the treatment groups.

The correlation between plasma prostaglandin type-2 expression (PGE, $PGF_{2\alpha}$ and PGE:$PGF_{2\alpha}$) and uterine fatty acid proportions (EPA, DHA, AA. and OA) were determined by the pearson moment correlation test. All statistical significance was at the $P<0.05$ level.

2.3 Result

2.3.1 The Effect of a High Fat and High Cholesterol Diet (HFHC) on the histology of the uterus

The histological analysis aimed to investigate whether exposure to a high fat and high cholesterol (HFHC) diet during pregnancy increases accumulation of lipid in the term or labouring rat uterus, this was determined indirectly by measuring the empty space or vacuoles within the uterine horn sections. The microscope images of uterine horn from rats fed either a HFHC or control diet during pregnancy are shown in Figure 3.6. The prepared microscope slides clearly show that uterine horn from non-labour HFHC rats has significantly more vacuoles or empty spaces within the myometrium when compared to both control non-labour and labouring group (**Figure 2.5**). Storing the samples in the ethanol prior to mounting the tissue was not ideal, but the empty spaces could potentially indicate that this is where fat deposits could have been. Moreover, the HFHC diet significantly increased the number of vacuoles ($P<0.01$) and mean area of vacuoles within myometrium when compared to control diet ($P=0.02$).

However, the type of delivery did not significantly affect the number vacuoles and mean vacuole area in both the HFHC and control myometrium.

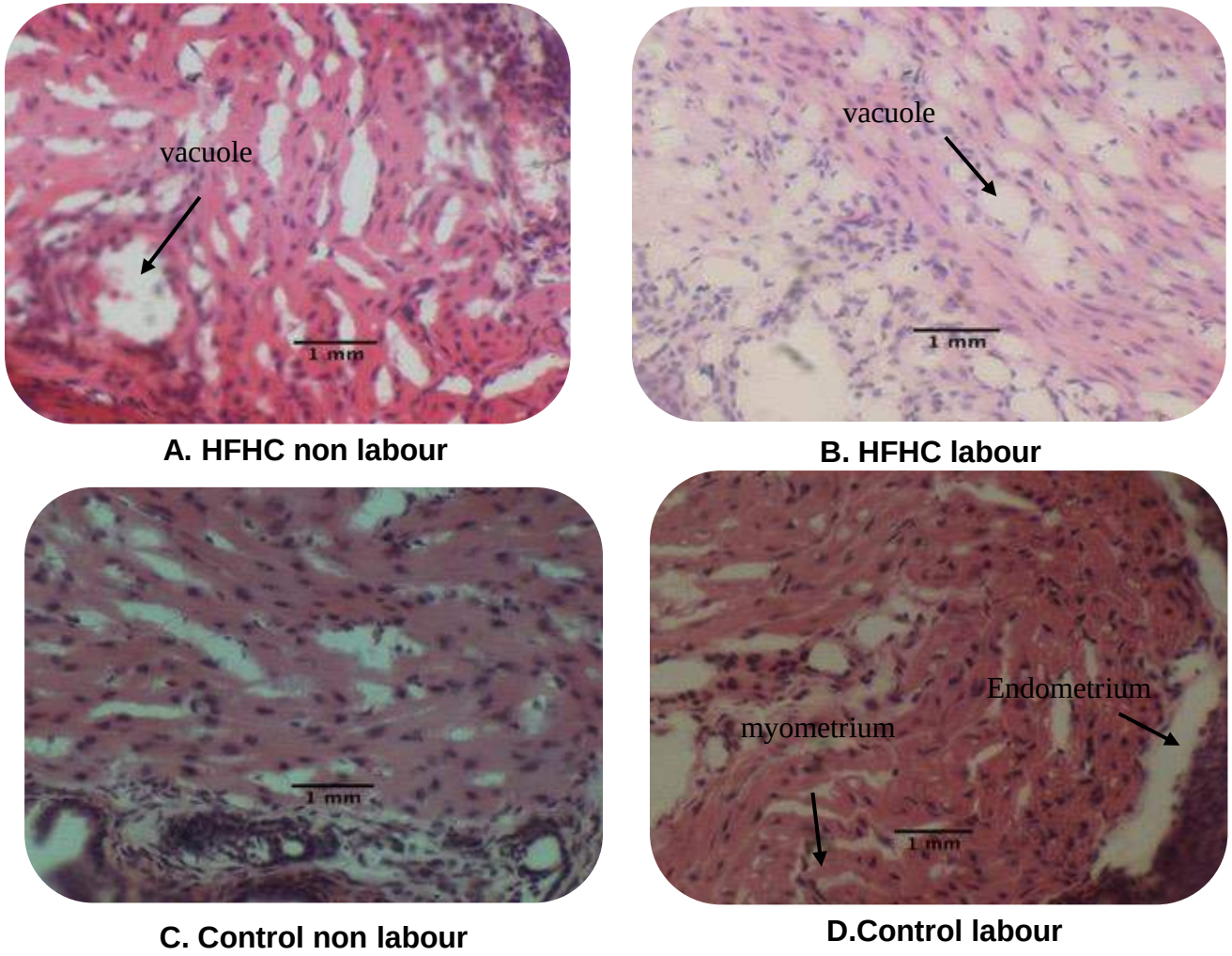

Figure 2.5 Term or labouring uterine histology from rats fed either a control or high fat and high cholesterol diet. The tissue stained with Haemoxillin and Eosin and observed in a light microscope with 20X magnification, Scale Bar 1 mm. The group of uterus tissue divided into 4 groups : (A) Control and non-labour, (B) Control labour, (C) HFHC non-labour, (D) HFHC labour.

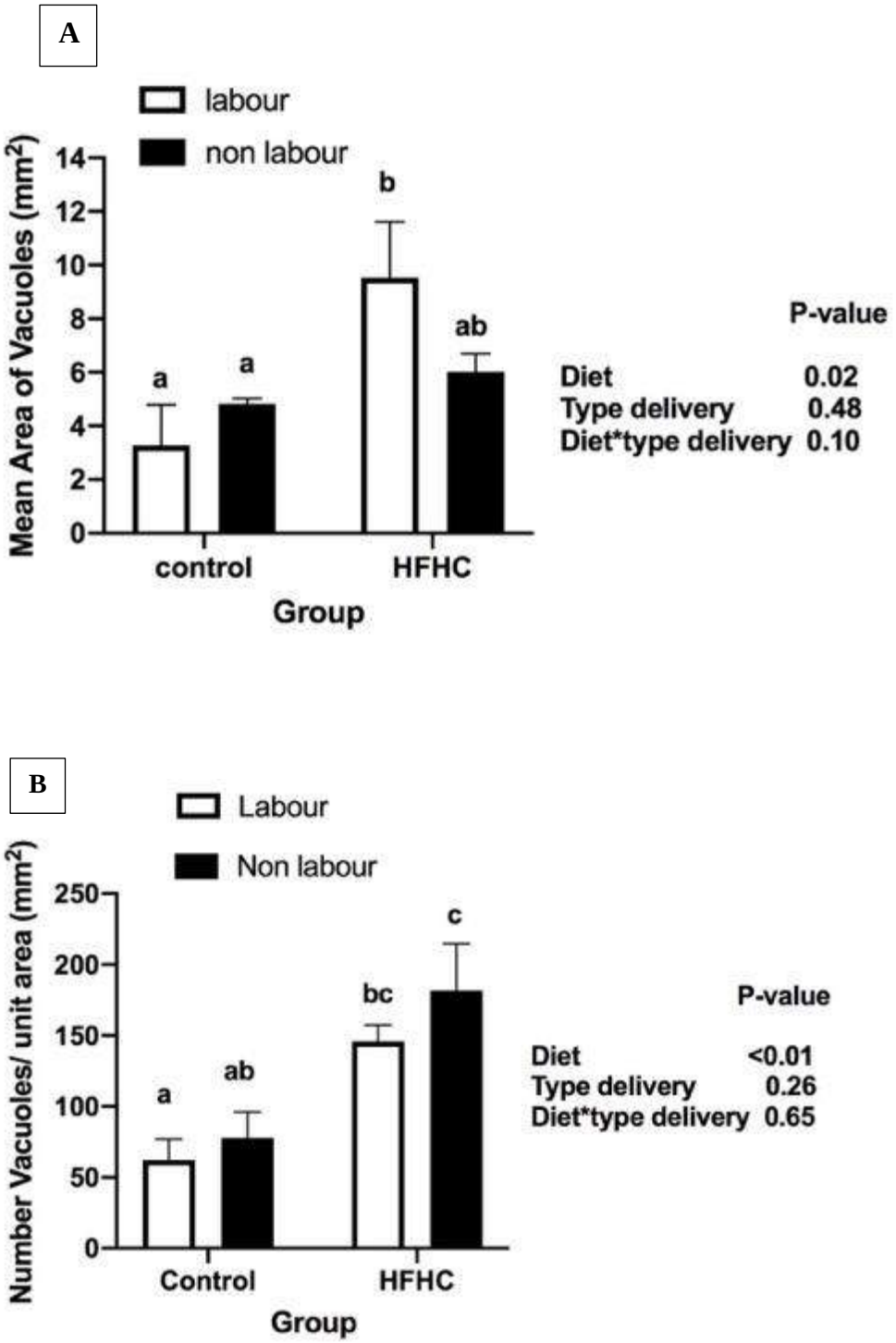

Figure 2.6 The effect of maternal diet (Con/HFHC) and stage of labour in (A) Mean area of vacuolae and (B) number of vacuoles per unit area (mm^2) of uterus tissue. The group of uterine tissues divided into four differents group: High fat and high cholesterol group (HFHC) non labour, High fat and high cholesterol group (HFHC) labour, Control labour and Control non labour. Values are means±SEM. Statistical analysis used two way ANOVA. Different superscript letters signify significant differences at the $P<0.05$

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2.3.2. Effect of Improving Diet at conception on fatty acid profile of the liver, plasma and uterus.

Analysis of the plasma fatty acid profile shows that exposure to a HFHC diet either pre-pregnancy or pregnancy alone significantly altered saturated, trans, omega-9, omega-7, omega-6 and omega-3 fatty acids (Table 2.4). The total saturated fatty acid content of the plasma (33.83±0.48%) decreased significantly in rats fed the HFHC diet in comparison to controls (34.46±0.53%) with P-value <0.01. Interestingly, the effect was reversed when rats fed the HFHC diet prior to pregnancy were switched to the control diet during pregnancy. The same was true for fatty acid proportion of total omega-6, LA, total omega-3 and ALA, DPA, DHA that showed a significant decreased in HFHC group, but then increased when HFHC fed rats were switched to control diet at post- conception (P<0.01). Conversely, HFHC diet significantly increased in HFHC diet, but then were significantly decreased when diet switched from HFHC to control diet (P<0.01) during pregnancy.

The fatty acid profile in the liver was very similar to the findings within the plasma (Table 2.5). Total saturated fatty acid in the liver of rats exposed to HFHC diet before pregnancy was 34.48±0.86 and during pregnancy was 36.60±0.86 which lower in comparison with rats exposed to control diet (43.44±0.95%) and HFHC-control groups (42.94±0.95%). However, the level of liver Monounsaturated fatty acid, omega-9 and OA in the HFHC group were 5-fold higher when compared to control and switching diet from HFHC to control diet during pregnancy significantly reversed the effect in HFHC-CON animals with P value <0.01. Total Hepatic omega-6 and individual omega-6 such as LA and AA were increased in rats exposed to control diet post-conception when compared with rats fed with HFHC diet during pregnancy.Liver Omega-3, including ALA, DPA and DHA were three times higher in control compared

than HFHC groups. Meanwhile, the level of ALA and DPA were not significantly different between CON-HFHC group with HFHC-CON.

The fatty acid composition of the uterus was only partially altered by the different dietary treatments before and during pregnancy (Table 2.6). There were no significant differences between saturated, monounsaturated, omega-9, omega-7 and omega-6 fatty acids between the 4 treatments group. However, total omega-3 contents in HFHC groups was 1.8-fold lower compared in the CON groups ($P<0.05$). Importantly, rats switched from HFHC to control diet has a similar amount of total omega-3 with the control group.

Plasma	Control (%) (n=5)	HFHC (%) (n=6)	Control-HFHC (%) (n-6)	HFHC-Control (%) (n=5)	P - value
	Mean±SEM	**Mean±SEM**	**Mean±SEM**	**Mean±SEM**	
Total Saturates	34.46±0.53^{ab}	33.83±0.49^{a}	35.52±0.49^{ab}	36.06±0.52^{c}	**0.01**
Total Trans	0.04±0.93^{a}	0.85±0.85^{b}	0.83±0.85^{b}	0.14±0.930^{a}	**<0.01**
C18:1 trans-9/ Elaidic acid	0.00^{a}	0.27±0.03^{a}	0.04±0.03^{b}	0.20±0.036^{a}	**<0.01**
C18:1 trans-11/ Vaccenic acid	0.040±0.08^{a}	0.62±0.05^{b}	0.10±0.05^{a}	0.00^{a}	**<0.01**
Total Monounsaturated	9.360±2.04^{a}	27.12±1.86^{b}	21.72±1.87^{b}	9.58±2.04^{a}	**<0.01**
Total Omega -9	7.600±0.58^{a}	23.55±0.53^{b}	18.98±1.66^{b}	7.74±0.58^{a}	**<0.01**
C18:1n-9/ OA	7.500±1.82^{a}	23.37±1.66^{b}	18.88±1.66^{b}	14.99±1.8^{a}	**<0.01**
Total omega 7	1.820±0.29^{a}	3.57±0.27^{b}	2.72±0.27^{ab}	1.84±0.29^{a}	**<0.01**
total omega 6	46.34±1.44^{b}	35.183±1.31^{a}	38.50±1.31^{a}	40.91±1.43^{b}	**0.03**
C18:2n-6/ LA	22.02±0.95^{b}	17.283±0.87^{a}	19.80±0.87^{ab}	19.18±0.94^{b}	**0.02**
C18:3n-6/ GLA	0.58±0.05	0.483±0.05	0.55±0.05	0.64±0.05	0.20
C20:2n-6/ Eicosadienoic acid	0.26±0.02	0.200±0.02	0.22±0.02	0.24±0.02	0.14
C20:3n-6/DGLA	0.46±0.04	0.483±0.03	0.40±0.03	0.44±0.04	0.34
C20:4n-6/ AA	18.40±1.00^{b}	11.567±0.92^{a}	13.32±0.92^{a}	19.62±1.00^{b}	**<0.01**
C22:5n-6/ DPA	3.48±0.48	4.467±0.43	3.48±0.43	4.10±0.48	1.19
Total Omega-3	9.700±6.63^{c}	3.033±0.61^{a}	5.32±0.61^{b}	9.00±0.66^{c}	**<0.01**
C18:3n-3/ ALA	1.12±0.09^{c}	0.367±0.08^{a}	0.71±0.08^{b}	0.84±0.09^{bc}	**<0.01**
C20:5n-3/ DPA	1.06±0.10^{c}	0.317±0.09^{a}	0.50±0.09^{ab}	0.76±0.10^{bc}	**0.02**
C22:6n-3/ DHA	7.42±0.5	2.250±0.51^{a}	4.02±0.51^{a}	7.20±0.55^{b}	**<0.01**

Table 2.4 The effect of feeding a CON (n=5) or HFHC (n=6) diet and reverse diet from CON to HFHC diet (n=6) or HFHC diet to CON (n=5) during pregnancy upon the maternal plasma fatty acid proportion. OA : Oleic Acid, LA: Linoleic Acid, GLA: Gamma Linoleic Acid, DGLA: Dihommo-gamma-linoleic acid, AA : Arachidonic acid, DPA : Decosapentaenoic acid, ALA : Alpha Linolenic acid, DHA : Decosahexaenoic acid. The values are means±SEM. Statistic analysis was used one way ANOVA Test, followed with LSD- post-hoc test with different superscript letters signify significant differences at the P≤0.05

Liver	Control (%) (n=5)	HFHC (%) (n=6)	Control-HFHC (%) (n-6)	HFHC-Control (%) (n=5)	P-value
	Mean±SEM	**Mean±SEM**	**Mean±SEM**	**Mean±SEM**	
Total Saturates	43.44±0.95^{b}	34.48±0.86^{a}	36.60±0.86^{a}	42.94±0.95^{b}	<**0.01**
Total Trans	0.02±0.09^{a}	0.83±0.08^{b}	0.82±0.08^{b}	0.26±0.09^{a}	<**0.01**
C18:1 trans-9/ Elaidic acid	0.02±0.03^{a}	0.32±0.03^{b}	0.22±0.03^{b}	0.09±0.03^{a}	<**0.01**
C18:1 trans-11/ Vaccenic acid	0.00±0.00^{b}	0.50±0.05^{a}	0.59±0.05^{a}	0.15±0.06^{b}	<**0.01**
Total Monounsaturates	6.92±2.06^{a}	31.02±1.88^{b}	21.17±1.88^{c}	7.00±2.06^{a}	<**0.01**
Total Omega -9	4.88±1.91^{a}	27.30±3.95^{b}	18.62±1.74^{c}	5.28±1.91^{a}	<**0.01**
C18:1n-9/ OA	4.56±1.90^{ab}	27.03±1.74^{b}	18.43±1.74^{b}	5.04±1.90^{c}	<**0.01**
Total omega 7	1.92±0.22^{a}	3.57±0.20^{b}	2.52±0.20^{ab}	1.66±0.22^{a}	<**0.01**
total omega 6	35.60±0.93^{b}	30.38±0.85^{a}	34.32±0.85^{b}	36.760±0.93^{b}	<**0.01**
C18:2n-6/ LA	13.08±0.70^{b}	15.65±0.64^{a}	15.90±0.64^{a}	11.840±0.70^{ab}	<**0.01**
C18:3n-6/GLA	0.58±0.11	0.48±0.10	0.47±0.10	0.580±0.11	0.81
C20:2n-6/ Eicosadienoic acid	0.44±0.05^{b}	0.23±0.04^{a}	0.28±0.04^{ab}	0.420±0.05^{b}	**0.01**
C20:3n-6/ DGLA	0.52±0.06	0.37±0.05	0.37±0.05	0.380±0.06	0.20
C20:4n-6/ AA	14.30±0.69^{c}	7.92±0.63^{a}	11.43±0.63^{b}	15.88±0.69^{c}	<**0.01**
C22:5n-6/ DPA	5.50±0.65	5.03±0.58	5.033±0.59	6.52±0.65	0.32
Total Omega-3	13.98±0.82c	3.30±0.75a	7.10±0.75b	13.02±0.82c	<**0.01**
C18:3n-3/ ALA	0.50±0.05	0.32±0.05	0.47±0.05	0.44±0.05	0.08
C20:5n-3/ EPA	0.18±0.02bc	0.07±0.02a	0.10±0.02ab	0.16±0.02b	0**.01**
C22:5n-3/DPA	1.14±0.08c	0.30±0.07a	0.58±0.07b	0.94±0.08c	<**0.01**
C22:6n-3/ DHA	12.18±0.75c	2.63±0.69a	5.97±0.69b	11.50±0.75c	<**0.01**

Table 2.5 The effect of feeding a CON (n=5) or HFHC (n=6) diet and reverse diet from CON to HFHC diet (n=6) or HFHC diet to CON (n=5) during pregnancy upon the maternal Liver fatty acid proportion. OA : Oleic Acid, LA: Linoleic Acid, GLA: Gamma Linoleic Acid, DGLA: Dihommo-gamma-linoleic acid, AA : Arachidonic acid, DPA : Decosapentaenoic acid, ALA : Alpha Linolenic acid, EPA : Eicosapentaenoic acid, DHA : Decosahexaenoic acid. The values are means±SEM. Statistic analysis was used one way ANOVA Test, followed with LSD- post-hoc test with different superscript letters signify significant differences at the P≤0.05

Uterus	Control (%) (n=5)	HFHC (%) (n=6)	Control-HFHC (%) (n-6)	HFHC-Control (%) (n=5)	P value
	Mean±SEM	**Mean±SEM**	**Mean±SEM**	**Mean±SEM**	
Total Saturates	40.62±4.09	46.07±3.73	45.23±3.73	44.66±4.09	0.78
Total Trans	0.54±0.19	0.93±0.17	1.07±0.17	0.42±0.19	0.06
C18:1 trans-9/ Elaidic acid	0.48±0.28	0.35±0.25	0.37±0.25	0.20±0.28	0.91
C18:1 trans-11/ Vaccenic acid	0.06±0.17[a]	0.58±0.15[c]	0.70±0.15[ab]	0.22±0.17[ab]	**0.04**
Total Monounsaturated	15.18±2.63	15.85±2.40	15.67±2.40	16.20±2.63	0.99
Total Omega -9	12.46±2.27	14.33±2.07	13.00±2.07	12.20±2.27	0.20
C18:1n-9/ \OA	12.40±2.23	14.15±2.03	12.83±2.03	12.12±2.23	0.90
Total omega 7	2.70±0.53	2.85±0.49	2.07±0.49	3.04±0.53	0.56
total omega 6	39.22±2.35	32.97±2.15	34.75±2.15	35.40±2.35	0.29
C18:2n-6/ LA	15.04±3.15	9.18±2.88	13.08±2.88	10.50±3.15	0.53
C18:3n-6/ GLA	2.16±0.43	1.75±0.39	1.53±0.39	1.54±0.43	0.69
C20:2n-6/ Eicosadienoic acid	0.86±0.10	0.62±0.10	0.62±0.10	0.76±0.10	0.30
C20:3n-6/ DGLA	2.40±0.55	2.05±0.50	2.25±0.50	1.62±0.55	0.76
C20:4n-6/ AA	12.48±1.92	12.25±1.75	11.25±1.75	13.72±1.92	0.82
C22:5n-6/ DPA	1.70±0.37	2.97±0.33	2.07±0.33	2.00±0.09	0.36
Total Omega-3	4.82±0.65[a]	2.80±0.28[b]	4.08±0.24[a]	4.24±0.48[a]	**0.02**
C18:3n-3/ ALA	0.93±0.35	0.60±0.70	1.05±0.35	0.60±0.50	0.86
C20:5n-3/ EPA	0.27±0.10	0.15±0.12	0.20±0.10	0.33±0.10	0.65
C22:5n-3/ DPA	0.90±0.13	0.83±0.12	0.98±0.12	0.80±0.14	0.74
C22:6n-3/ DHA	3.04±0.40	1.817±0.37	2.3±0.37	3.16±0.40	0.10

Table 2.6 The effect of feeding a CON (n=5) or HFHC (n=6) diet and reverse diet from CON to HFHC diet (n=6) or HFHC diet to CON (n=5) during pregnancy upon the maternal uterus fatty acid proportion. OA : Oleic Acid, LA: Linoleic Acid, GLA: Gamma Linoleic Acid, DGLA: Dihommo-gamma-linoleic acid, AA : Arachidonic acid, DPA : Decosapentaenoic acid, ALA : Alpha Linolenic acid, EPA : Eicosapentaenoic acid, DHA : Decosahexaenoic acid. The values are means±SEM. Statistic analysis was used one way ANOVA Test, followed with LSD- post-hoc test with different superscript letters signify significant differences at the $P \leq 0.05$

2.3.3 The Effect of the HFHC diet and dietary switch at conception on plasma prostaglandin concentrations during labour

The prostaglandin analysis identified that $PGF_{2\alpha}$, PGE_2 and the ratio of PGE_2: $PGF_{2\alpha}$ were not significantly different across the dietary groups (Figure 2.7). Moreover, there was no correlation between the proportion of EPA and DHA in the uterus with plasma concentration of prostaglandin $PGF_{2\alpha}$, PGE_2 and the PGE_2: $PGF_{2\alpha}$ ratio (Figure 2.8 and Figure 2.10). Furthermore, there was no correlation between uterus fatty acid proportion (EPA, DHA , AA and OA) with prostaglandin production in the plasma of control group ($P>0.05$). The same result occurs in HFHC group that showed no correlation between proportion of EPA, DHA, AA and OA in the uterus with prostaglandin type 2 production in plasma (Table 2.7).

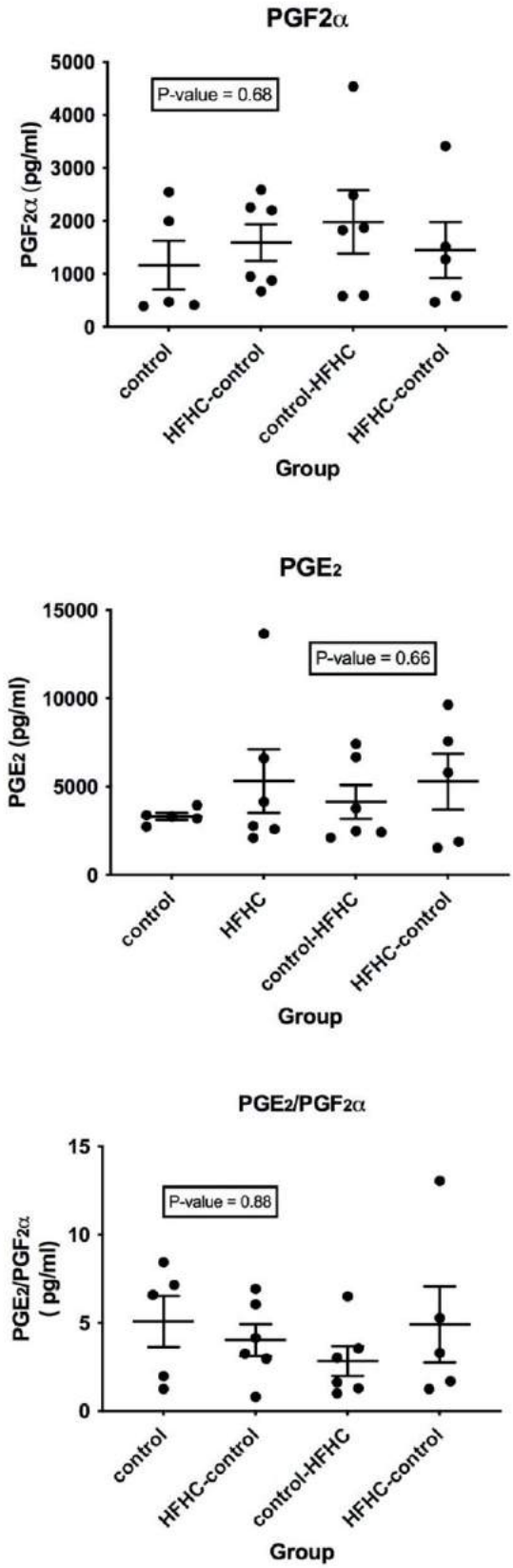

Figure 2.7 The effect of Control or HFHC diet and switch at conception on plasma concentration of the 2 series prostaglandins (pg/ml) ; (A) $PGF_{2\alpha}$, (B), PGE_2 and (C), PGE_2: $PGF_{2\alpha}$. Values are mean±SEM

Fatty acids in the uterus	PGE_2		$PGF_2\alpha$		PGE_2:$PGF_2\alpha$	
	Coefficient (r)	P-value	Coefficient (r)	P-value	Coefficient (r)	P-value
Control group						
EPA	-0.17	0.64	0.21	0.56	-0.29	0.41
DHA	-0.21	0.55	-0.04	0.92	0.25	0.49
AA	-0.04	0.92	0.55	0.10	-0.74	0.06
OA	0.15	0.68	-0,58	0.08	0.44	0.20
HFHC group						
EPA	-0.15	0.67	0.42	0.20	-0.12	0.73
DHA	0.42	0.20	-0.23	0.51	0.43	0.20
AA	-0.39	0.24	-0.39	0.24	0,17	0,62
OA	-0.27	0.42	0.41	0.21	-0.21	0.57

Table 2.7 The correlation between uterus fatty acid and plasma prostaglandin production in both control and HFHC group. The control group refers to control diet and HFHC-CON diet, while HFHC group refers to HFHC diet and CON-HFHC diet. Pearson Moment Correlation was used to analysed the correlation between fatty acid proportion in the uterus ; Eicosapentaenoic acid (EPA), Decosahexaenoic acid (DHA), Arachidonic acid (AA) and Oleic acid (OA) and Prostaglandin E_2 (PGE_2), Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and the ratio between PGE_2 and $PGF_2\alpha$ with signify significant $P<0.05$)

2.4 Discussion

The evidence from our animal study suggests that high fat diet might increase accumulation of lipid in the myometrium of obese pregnant rats which can be seen from increased mean area and number of vacuole/unit area of the uterus after H&E staining. Fixing the tissue in the ethanol possibly extracted lipid from the tissue during tissue processing, therefore, they appear as empty spaces after staining with H&E. The empty spaces in the myometrium tissue are potential indicators of where fat deposits may have been.

The uterus is an organ where lipid distribution plays a critical role in its function. Many studies illustrated the importance of cholesterol as part of the cell membrane and its role in decreasing membrane fluidity as well as controlling cell membrane permeability. Cholesterol is also a direct precursor of the steroid hormones

including oestrogen and progesterone (Bartels and O'Donoghue, 2011). A high fat diet increases fat storage and decreases cholesterol in the liver within the presence of Liver X receptor (LXR), a receptor that regulates cholesterol, fatty acid and glucose homeostasis (Kalaany et al., 2005). The study proved that LXRβ prevents accumulation of cholesteryl esters in mouse myometrium by controlling a gene that regulates cholesterol efflux and storage (Mouzat et al., 2007). The same study also proved that lack of LXR increases lipid accumulation and causes muscular defect in the uterine smooth muscle, resulting in a decreased amplitude of uterine contraction in mice. A similar mechanism of increased fat storage following exposure of HFHC might occur in uterus of pregnant rat fed with HFHC diet during pregnancy, which may potentially inhibit myometrium contractility during parturition.

There is evidence that increased dietary fatty acid or free fatty acid content increases intramyocellular lipid in skeletal muscle tissue, suggesting that skeletal muscle stores fat when fatty acids are high (Schrauwen et al., 2010). A similar mechanism might occur in uterine tissue, especially the myometrial layer. Obese pregnant women exhibit insulin resistance and have higher plasma levels of insulin compared to normal weight pregnant women. This condition would be expected to decrease lipolysis in adipose tissue and stimulate hepatic FFA uptake, then it possibly stimulates fat storage, including triglyceride in the myometrium (Herrera and Ortega-Senovilla, 2010). Increased fat storage, especially triglyceride in the myometrium is associated with decreased muscle content, which could be the mechanism behind prolonged and dysfunctional labour often observed with maternal obesity (Gam et al., 2017). However, the current study did not identify triglyceride location and levels within the myometrium, so, future work would be required to confirm this theory.

Importantly, our findings agree with the hypothesis that diet affects the fatty acid profile of the plasma, liver and uterus. Rats fed a HFHC diet had a lower n-3:n-6 ratio in the plasma and liver compared to controls. This study also provided nice evidence that improving diet from a HFHC at conception to a control chow diet reverses the fatty acid composition in plasma, liver and uterus and increased potential to produce prostaglandins to improve myometrial contractile function.. Rats that were exposed to the HFHC diet in preconception then switched to control diet post-conception shows a significant increase in total saturates, total omega-3, ALA, EPA, total omega-6 and arachidonic acid, but decreased total monosaccharide, total omega-9, and OA in plasma and liver. The liver fatty acid profile suggests that hepatic fatty acid profile in the rat feeds HFHC obesogenic before pregnancy can be regenerated by switching Control diet at conception. In addition, This result is consistent with Raatz et al. (2001) that showed a higher number of saturated fatty acid, total n-3, arachidonic acid, DPA and DHA in plasma and lower EPA profile in the low fat diet compared with high fat diet.

One interesting finding in this study is switching diet from HFHC diet to control diet only affected omega-3 in the uterus. This result suggests that the uterus is more resistant to change in fatty acids similar to the brain (Murphy, 1990). Moreover, our study was conducted in the rat where uterine tissues were collected during established labour, and important to note that the fatty acids might have been used during the labour process, such as use in prostaglandin production. Therefore, the differences in fatty acid composition might only be identified before parturition (Muir et al., 2018). This theory is supported by other studies that identified release of arachidonic acid near term and activation of pro-inflammation cytokine (TNF-α and IL-β) during the last trimester induces prostaglandin production.

The current study identified that switching from a HFHC to control diet during pregnancy is not associated with plasma changes in prostaglandins (PGE_2 and $PGF_{2\alpha}$). Furthermore, it also provided evidence that there was no correlation between the proportion of EPA, DHA, AA and OA and plasma type 2 prostaglandin production in both the control and the HFHC group. This study contrasts with Cheng et al. (2015)'s study that showed a decreased production of $PGF_{2\alpha}$ in the maternal endometrium of ewes after OA supplementation during pregnancy. The findings are also in contrast to Roman et al. (2006) where it was found that DHA inhibits prostaglandins production by up to 80% in comparison with the control group and was more potent in supressing $PGF_{2\alpha}$ and PGE_2 as compared to EPA in cultured decidual cells under inflammation. The potential of PGE_2 and $PGF_{2\alpha}$ to quickly convert to PGEM and PGFM might be the reason behind similar PGE_2 and $PGF_{2\alpha}$ concentrations between the control and HFHC group. This result is consistent with the systematic study conducted by Wood et al. (2021) that reported that the pattern of plasma PGE_2 and $PGF_{2\alpha}$ production is not consistently altered at term labour.

2.5. The strength and limitation of study

The current study has identified that a high fat diet increased mean area and number of empty spaces or vacuoles potentially indicating where the lipid may have been stored after H&E staining. A good future study would be to officially confirm lipid accumulation in the myocytes of the uterus by using oil red o staining which potentially leads to myometrial contractile defects. This could be a key mechanism related to the prolonged and dysfunctional labour often observed with maternal obesity (Mouzat et al., 2007).

This study also proved that change of diet during pregnancy significantly improved the fatty acid composition of the plasma, liver and uterus. However, this

study only investigated the proportion of fatty acids from the total fat so did not calculate absolute fatty acid concentrations in the plasma, liver and uterus. A useful study in the future would be to analyse the actual concentration of fatty acids to prove that a high fat, high cholesterol diet not only decreases the proportion of omega-3 but also the level of omega-3 fatty acids in the uterus.

The current evidence identified that fatty acid have no corelation wit prostaglandin type-2 in control and HFHC group. $PGF_{2\alpha}$ and PGE_2 are ideally measured at their site of synthesis because of rapid metabolism. $PGF_{2\alpha}$ and PGE_2 are quickly converted into PGFM and PGEM. Some studies have reported that analysis of prostaglandins in peripheral plasma based upon their metabolites PGFM and PGEM concentrations are consistent indicators of uterine prostaglandin production (Heuwieser et al., 1993). This theory is supported by another study that reported estrogen upregulates COX-2 expression in the maternal endometrial tissue and associated with PGDH expression leading to increased $PGF_{2\alpha}$ output to PGFM in late-onset labour (Smith, 2001). Therefore, future studies will need to investigate the effect of HFHC diet on PGFM and PGEM which are more accurate in observing prostaglandin concentrations compared to $PGF_{2\alpha}$ and PGE_2 at the onset of labour.

3. Effect of high fat high cholesterol diet on fatty acid composition and protein expression of PPARs and aromatase in the rat placenta.

3.1 Introduction

Many studies have established the link between maternal obesity and increased risk for prolonged and dysfunctional labour in both humans and animals (Bogaerts et al., 2013; Carlson et al., 2015; Muir et al., 2016). Although the mechanism behind prolonged and dysfunctional labour with maternal obesity remains unclear, fatty acids are thought to play a pivotal rule in the timing and length of parturition (Cheng et al., 2015; Elmes et al., 2004; Hag et al., 2019; Harper et al., 2010). Recent research has reported that omega-3 supplementation decreases prostaglandin production in cultured decidual cells from sheep providing evidence that omega-3 PUFAs may have the potential to decrease the risk of preterm birth (Baguma-Nibasheka et al., 1999; Roman et al., 2006). Meanwhile, supplementation of high levels of OA increase PGE2:PGFα production in the maternal endometrium of late gestation sheep suggesting that OA could both improve and inhibit myometrial contractions during parturition (Cheng et al., 2015). In contrast to this, increases in dietary consumption of omega-6 increase placental prostaglandin synthesis increasing the risk of preterm birth (Elmes et al., 2004).

Placentae from obese pregnant women have been identified to accumulate greater saturated lipid levels compared to pregnant lean women (Saben et al., 2014). Dietary HFHC at conception increased the number and size lipid droplets in the placenta of pregnant rat (Luwagie et al., 2018) . This lipid accumulation is caused by changes in placental fatty acid handling, such as fatty acid uptake and decreased fatty acid oxidation

(Calabuig-Navarro et al., 2016; Lassance et al., 2015). In addition, Maternal obesity markedly increased cholesterol and triglyceride concentration in maternal and foetal blood and it is associated with increased protein and mRNA expression of PPAR γ in ctyledonary ewes in midgestation Zhu et al., (2010)

Another isoform PPAR δ is highly expressed and distributed in the placenta (Wagner and Wagner., 2010). Immunocytochemistry staining results shows the presence of PPAR α, δ and PPAR γ to be specific to the trophoblast layer of the human chorionic villi (Hadschuh et al., 2009; Wang et al., 2002). PPARα functions to regulate fatty acid metabolism, while PPAR δ is involved in cell differentiation and lipid accumulation and PPAR γ plays a pivotal role in adipogenesis, inflammation and glucose metabolism (Duttaroy, 2009; Peng et al., 2021).

Lappas et al., (2002) mentioned that PPAR γ has an important role to secrete IL-6, IL-8 and TNF- α in human placenta. From the same study also explained that adding 30μM PPARγ activator into human placenta tissue decreases IL-8 and TNF- α released from human placenta and suppresses Nf-kB DNA-binding activity from human placenta, amnion and chorion. Moreover, many studies reported that decreased activity of Nf-kB reduce the expression of protein MMP-9 which has an important role to regulate extracellular matrix (ESM) degradation prior to parturition in human placenta cell and myometrium at term labour (Borel et al., 2008; Choi et al., 2007; Xu et al., 2002 ; Rajabi et al., 1990). Therefore, increased PPAR γ in maternal obesity could be the mechanism behind prolonged and dysfunctional labour through pro-inflammatory cytokines and MMP suppression.

Another possible mechanism related to role PPAR γ affect parturition is related to COX-2 expression since Immunohistochemical study proved localization of PPAR γ expression in the human placenta before active labour, but then significantly reduce during

labour and that its repression is associated with an increase of COX-2 (Albanese et al., 2004). The promoter region of COX-2 contains a response to element for PPARs indicating PPARs can directly influence transcription of the COX-2 gene (Subbaramaiah et al., 2001). Moreover, the evidence shows that knockout PPAR α stimulates Th-2 cytokine including IL-4 and IL-10 mRNA and increase protein COX-2 where this could stimulate contractions in the mice during labour(Yessoufu et al., 2006; Borel et al., 2008). On the other hand, increasing mRNA PPAR δ at term labour associate with increasing 15-delta Prostaglandin J2 in the human amnion where 15-delta Prostaglandin J2 also acts as natural ligand of PPAR γ that can inhibit IL-1ß and induce COX-2 synthesis (Berry et al., 2003; Sawano et al., 2002; Borel et al., 2008). This is an important finding as it has been reported that COX-2 suppression delays labour in both human and animal pregnancies (Fournier et al., 2007; Schuler et al., 2006).

Despite evidence to suggest that PPARS might play a pivotal roles in prolonged and dysfunctional labour associated with maternal obesity, another mechanism could be involved related to synthesis of the pregnancy related hormones progesterone and estrogen (Mueller et al., 2006; Schoppee et al., 2002). Progesterone does not decrease dramatically at term in human pregnancy, but it gradually falls while estriol production accelerates, leading to a higher oestrogen, progesterone ratio. However, Muir et al. (2016) reported that progesterone was significantly higher at term in the obese rat but falls dramatically with onset of labour and shown to be similar to the concentrations in labouring control rats. These findings suggest that there are differences in oestrogen synthesis between HFHC and control fed rats which may be associated with the unsynchronised contractions in the obese HFHC fed rats. This theory supported by many studies that reported the increasing production of oestrogen associates with enhance of prostaglandin and oxytocin and MLCK that could lead to stimulate contraction during labour (Kota et al., 2013).

Aromatase is a crucial enzyme that controls synthesis of oestrogen. (Mueller et al., 2006). In the human placenta, androgen derived from maternal and foetal adrenal glands are converted into oestradiol, specifically androstenedione converted into estrone by the enzymatic action of placental aromatase (Crajka-Oraniec and Simpson., 2010). However, the activity of aromatase is downregulated by PPAR γ in human ovarian granulose-like tumour cell line and mouse ovary (Fan et al., 2005). Furthermore, some studies have proposed that PPARs might impact on aromatase and steroidogenesis in human and animal study (Komar et al., 2005).

Based on the evidence provided above, some PUFAs such as DHA,EPA and AA are natural ligands for PPARs (Grygiel-Górniak, 2014b). EPA and AA are efficient activators of PPAR α and PPARδ, but display only little activation on PPAR γ (Forman 1997), therefore any differences in the level of fatty acids within CON and HFHC placentas could potentially alter PPAR expression. Moreover, aromatase is an important enzymes for oestrogen production that also affected by PPARs expression, so that change of PPAR expression due to high fat diet might contribute to aromatase expression.

3.1.1 Objective and Hypothesis

The aim of this study was to investigate the effect of a HFHC diet on the fatty acid proportion and protein expression of PPARs and aromatase in the placenta. The hypothesis of this study was that the HFHC diet will alter fatty acid composition of the placenta and increase placental expression of PPARs . The increased expression of PPAR will suppress expression of aromatase in the placenta and potentially to increase the risk of prolonged and dysfunctional labour with maternal obesity.

3.2 Materials and Method

3.2.1 Animal Experiment

Details on the animal trial for this experiment is already detailed in Section 3.2.1. The rats that used in this experiment were the same as Chapter 3. After delivery of the 5th pup, each rat dam was immediately euthanised by CO_2 asphyxiation and cervical dislocation. The placenta was dissected out, weighed and snap-frozen with liquid nitrogen and stored at -80°C until analysis.

3.2.2 Placenta lipid Extraction

Frozen placental tissue weighing 300 mg was crushed under liquid nitrogen with a pestle and mortar. Once crushed it was homogenised in 2 ml of ice-cold 0.9% saline in 30 seconds followed with vortexed in 3 ml of 99.9% Propan-2-ol and then incubated at room temperature for 5 minutes. After incubation, 6 ml of 98% chloroform was added to the solution, inverted and centrifuged at 3000rpm for 10 minutes. Then, within a fume hood, transfer the bottom layer of chloroform into the solvent-resistant plastic tube using a disposable glass pipette taking care not disturb the other layers when extracting the target layer. The solvent solution was then dried down under nitrogen in the fume hood at 2psi, and the temperature was 37°C until the solution evaporated. After the solution was evaporated, 3 ml of hexane was added then kept the tube containing lipid and hexane at -20°C until FAME analysis.

3.2.3 Fatty Acid Methyl Esters (FAME) of Placenta Tissue

The samples containing 3 ml of lipid and hexane were evaporated carefully under nitrogen in a fume hood , then added 0.2 ml hexane followed with 0.7 ml of 10M Potassium Hydroxide (KOH) and 5.3ml Methanol. The samples containing both KOH and methanol were then vortexed for 2 minutes. After that, the sample was then

incubated in the water bath at 55°C for 90 minutes vortexing every 20 minutes for 5 seconds. The samples were then cooled down in cold water for 10 minutes, then 0.58ml of 12M sulphuric acid was added. The samples containing sulphuric acid were then mixed by vortex, and incubated again for 90 minutes, with vortexing every 20 minutes. After 90 mins incubation the samples were cooled again in a water bath for 55°C in 10 minutes. Once cooled, 3 ml of hexane was added, each sample vortexed and mixed for 30 seconds. Samples were then centrifuged for 5 minutes at 2000rpm. The resulting top layer of hexane was then transferred into a solvent-resistant tube and kept at -20°C until Gas chromatography (FID) analysis.

3.2.4 Gas Chromatography analysis

Gas chromatography was used to separate and detect the ions from a mixture of FAMEs. The FAME samples were removed from -20°C storage then vortexed to resuspend the lipid in hexane. Samples were then dried down under nitrogen and resuspended in 1 ml hexane to increase the fat concentration.

The Gas chromatography (GC) vials (2 ml crimp top vials, Thermo Scientific) were labelled appropriately, and 1 µl of each sample was pipetted into each sample, followed with tight sealing of the lid. All samples were then loaded into the GCFID machine sample carousel (Perkin Elmer Clarus 580) with capillary column Cp-Stll 88 v2 50m column 0.25mm ID, 0,2um film thickness, injector volume 1µl. The injector temperature and GCFID temperature was 260°C, FID setting H2-45ml/min with hydrogen flow rate approximately 3ml/min. The gas chromatography analysis was optimised and maintenance by Nutritional Science technician, Jon Stubberfield.

Gas Chromatography methods used to separate and detect molecules from a mixture of FAME. A small amount of sample, approximately 1µl injected mechanically

into one end column, then will separate into an individual compound with the help of helium as intermediate gas. Each component characterised based on its retention time and the area under each peak indicates the relative quantities. The Principle of GC FID presented in Figure 2.3 in Chapter 2.

3.2.5 Tissue preparation

Rat placenta tissues approximately 300 mg in weight were, then crushed under liquid nitrogen followed with homogenised in the presence of 2 ml ice-cold extraction buffer (32.5 mM Tris/HCL, 3.08 mM EDTA, pH 7.5) and protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA) for 30 second pulses. Homogenate for PPARs and aromatase underwent centrifugation in 3500 g for 10 min at 4°C to isolate the supernatant. Approximately 1.5 ml of supernatant was then extracted and separated into 2 different tubes which 0.5 ml was then added with the same amount of 2XSDS (0.5 ml) and stored at -20°C and the other 1 ml supernatant was stored at -20°C prior to protein quantification.

3.2.6 Protein Assay

A protein assay was used to determine the total protein concentration of the supernatant before use for western blot analysis. The standard protein concentrations were diluted between 0 to 1.2 mg/ml from 5 mg/ml BSA in ionic water. The protein analysis began with set up 50 µl blank and diluted supernatant (20X, 30X, 50X) within a 96 well plate. Each sample was measured in duplicate. The next step was addition of 150 µl 0.1M NaOH followed by 50µl solution 1 (5 ml 2%(w/v) Na2CO3 in 0.1M NaOH, 0.5ml 1% (w/v) $CuSO_4$ and 0.5ml 2% (w/v) KNa Tartrate then, it was left for 5 minutes. Next step 50µl solution 2 was added (5 ml 0.1M NaOH and 0.5 ml Folin's

Clocalteu's reagent) and left for a further 20 minutes. The final step was to read the absorbance at 550nm using a plate reader (Tecan Grp Ltd, Switzerland). The standard curve was then used to determine the protein concentration of the unknown placental samples.

3.2.7 Western blot analysis

Samples were diluted with 1X Sodium dodecyl sulphate mix to a total concentration of 5µg/µl. Samples were then boiled at 100°C for 5 minutes, and centrifuged at 13000g for 3 minutes. Next 15 µl protein samples were loaded into the wells, then electrophoresed at 200 V for 45 minutes. The protein was then transferred onto nitrocellulose membrane (Hybond-C Extra, Amersham Bioscience) within an ice-cooled transfer buffer solution at 50 A for 2 hours. The transfer buffer solution (400mM Glycine, 30mM Tris Base and 5% isopropanol) was loaded until to the top of the tank, and a flea included to stir the transfer buffer solution. Next the excess membrane was cut and protein ladder marked. The Nitrocellulose Membrane was then washed with Ponceau-S stain to confirm protein transfer to the membrane. The membrane was then blocked with 5% milk in 1X Tris Buffered Saline- Tween (TBST) (10% TBS, 1% Tween 20) for 1 hour at room temperature. After blocking with milk, the membrane was washed with 1XTBST for 30 minutes (5 minutes X 6 wash cycle using 1X TBST). Then, the membrane was blotted overnight at 4°C with anti-mouse primary antibody IgG (1:2000 for PPARα,1:1000 for PPARδ PPAR γ and Aromatase) (Abcam, UK) . Next, the membrane was washed for 1 hour with TBST solution, and the solution replaced every 5 minutes to reduce the unspecific binding of the primary antibody. Following this wash cycle, the membrane was blotted with anti-rabbit secondary antibody IgG Horseradish peroxide for 1 hour for aromatase and 1.5 hours for PPAR

α, δ andγ respectively. Secondary antibody was diluted in 5% milk with 1.2% tween 20 in TBST to reduce unspecific binding of the secondary antibody and got clear protein band. Secondary antibody dilutions of PPAR α, PPAR δ, PPAR γ and Aromatase were diluted with 5% milk with ratio 1: 30.000, 1;40.000, 1:40.000 and 1:15.000 respectively. The membrane was then washed for 1 hour with TBST solution (replaced TBST every 5 minutes to reduce unspecific binding of the secondary antibody and subsequent background exposure). The membrane was incubate with ECL normal detection reagent (GE Healthcare UK ltd) for 5 minutes for aromatase and incubate 2 minutes with ECL select (GE Healthcare UK ltd) for PPARs at room temperature to initiated the chemiluminescent reaction. The membrane was then exposed to photogenic film (GE Healthcare UK Ltd) under red light to capture the photons emitted from the chemiluminescent reaction. Following this, the film was developed and fixed. Protein expression was quantified using a Bio-Rad Gel-Doc analysis system employing quantity one analysis software (Bio-red USA).

The last step was re-probing blots with β-actin (Sigma-Aldrich; primary antibody 1:1000 and secondary antibody 1:40,000) to verify equal protein loading. If the protein of interest was the same molecular weight as β-actin, membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo-Fisher Scientific) for 30 min, respectively, followed with the same method stated previously for western blotting.

3.2.8 Statistic analysis

All statistical analysis was carried out using SPSS version 24 software and expressed as the mean value ± standard error mean (SEM) where P=<0.05 was considered statistically significant. The homogeneity of the data was assessed and if not

normally distributed, the data was suitably transformed with Log 10 to get a normal distribution. Independent T-test was used to determine any significant differences of rat placenta fatty acids composition, PPARs and aromatase expression between control and HFHC group.

3.3. Result

3.3.1 Effect of high fat and high cholesterol diet on fatty acid composition of the rat placenta.

The fatty acid composition of the placenta was significantly affected by the HFHC diet (Table 3.1). HFHC diet significantly decreased the total saturated fatty acids in the placenta from 69.87±0.29% to 58.71±0.43% ($P<0.01$). Exposure to the HFHC also significantly increased tridacylic, myristic acid, pentadecylic acid proportion ≥ 1.5-fold when compared to placentas from rats fed a control diet. However, The level of stearic and arachidic acid was significantly lower in the HFHC group when compared to controls ($P<0.05$). Interestingly, there was no significant difference between the placental level of total omega-3 and omega-9 fatty acids between HFHC and control fed rats. In contrast, the omega-6 fatty acids were affected by diet where the proportion of linoleidic acid significantly decrease in HFHC diet with $P =0.03$. In contrast, exposure to the HFHC diet significantly increased percentage of placenta DGLA with three fold higher compare than control fed diet ($P=0.01$).

Fatty acid	**Control (n=5)** Mean±SEM (%)	**HFHC (n=4)** Mean±SEM (%)	**P-value**
Saturated fatty acid			
Total Saturated	69.87±0.32	58.71±0.43	**<0.01**
C4:0/ Butyric acid	0.17±0.68	0.32±0.05	0.28
C6:0/ Caproic acid	0.17±0.10	0.05±0.01	0.2
C8:0/ Caprylic acid	0.08±0.05	0.08±0.01	0.81
C10:0/ Capric acid	0.10±0.02	0.19±0.02	**0.03**
C11:0/ Undecylic acid	0.11±0.04	0.17±0.04	0.37
C12:0/ Lauric acid	1.36±0.06	1.47±0.10	0.4
C13:0/ Tridecylic acid	0.04±0.01	0.10±0.01	**0.02**
C14:0/ Myristic acid	1.41±0.06	2.67±0.38	**0.04**
C15:0/ Pentadecylic acid	0.79±0.03	1.13±0.06	**<0.01**
C16:0/ Palmitic acid	31.09±1.16	28.73±1.68	0.32
C17:0/ Margaric acid	0.76±0.04	0.76±0.03	0.98
C18:0/ Stearic acid	30.04±0.88	19.94±1.46	**<0.01**
C20:0/ Arachidic acid	0.67±0.031	0.43±0.05	**<0.01**
C21:0/ Heneicosylic acid	0.24±0.08	0.04±0.01	0.06
C22:0/ Bahenic acid	0.72±0.07	0.58±0.09	0.21
C23:0/ Tricosylic acid	0.14±0.02	0.19±0.01	0.21
C24:0/Lignoceric acid	1.95±0.21	1.70±0.27	0.66
Monounsaturated			
Total Monos	0.69±0.12	1.07±0.43	0.13
C14:1/myristic acid	0.12±0.04	0.25±0.03	0.09
C15:1/ Pentadecenoic acid	0.06±0.011	0.06±0.01	0.98
C16:1n-7/ Palmitic acid	0.04±0.02	0.24±0.05	**0.01**
C17:1/ Heptadecenoic acid	0.25±0.01	0.43±0.09	0.29
C22:1n-9/ Erucic acid	0.03±0.02	0.06±0.01	0.27
C24:1/ Nervonic acid	0.21±0.06	0.10±0.04	0.26
Omega-9			
Total Omega 9	0.53±0.08	0.33±0.03	0.06
C18:1n-9/ OA	0.47±0.10	0.26±0.03	0.21
Omega-6			
Total Omega-6	1.17±0.12	1.37±0.09	0.32
C18:2n-6, 9 All-trans/ Linolelaidic acid	0.46±0.10	0.18±0.02	**0.03**
C18:2n-6/ LA	0.13±0.02	0.07±0.02	0.09
C20:2n-6/ Eicosadienoic acid	0.19±0.04	0.09±0.02	0.11
C20:3n-6/ DGLA	0.22±0.27	0.85±0.16	**0.01**
C20:4n-6/ AA	0.15±0.05	0.13±0.06	0.89
Omega-3			
Total Omega 3	0.96±0.12	0.82±0.14	0.52
C18:3n-3/ ALA	0.45±0.11	0.44±0.08	0.94
C20:3n-3/ Eicosatrienoic acid	0.28±0.07	0.24±0.03	0.71
C20:5n-3/ EPA	0.08±0.03	0.05±0.02	0.51
C22:6n-3/ DHA	0.15±0.03	0.09±0.03	0.21

Table 3. 1 The comparison of the fatty acid profile of placenta from rats fed either a control or HFHC diet. OA : Oleic Acid, LA: Linoleic Acid, DGLA: Dihommo gamma linoleic acid, AA : Arachidonic acid, ALA : Alpha Linolenic acid, , EPA : Eicosapentaenoic acid, DHA : Decosahexaenoic acid. Values are means±SEM as a percentage of total fatty acids. It is important to note that the fatty acids do not add up to 100% as between 30-40% of fatty acids were unknown following analysis by GC-FID . Statistical analysis was by independent T-test, at the $P \leq 0.05$ level.

3.3.2 Effect of High Fat and High Cholesterol Diet in PPARs and Aromatase In Placenta

The western blot study was used to investigate the impact of exposure to a HFHC diet during pregnancy on PPARs and aromatase expression. From the current study, exposure to the HFHC diet during pregnancy significantly altered PPAR expression (Figure 3.1), but interestingly, did not significantly affect aromatase expression ($P>0.05$) (Figure 3.2). Exposure to the HFHC diet significantly increased protein expression of PPARγ ($P=0.01$) about 30% within the placentae when compared to CON. However the expression of PPARα and PPAR δ were not significantly different between CON and HFHC diet.

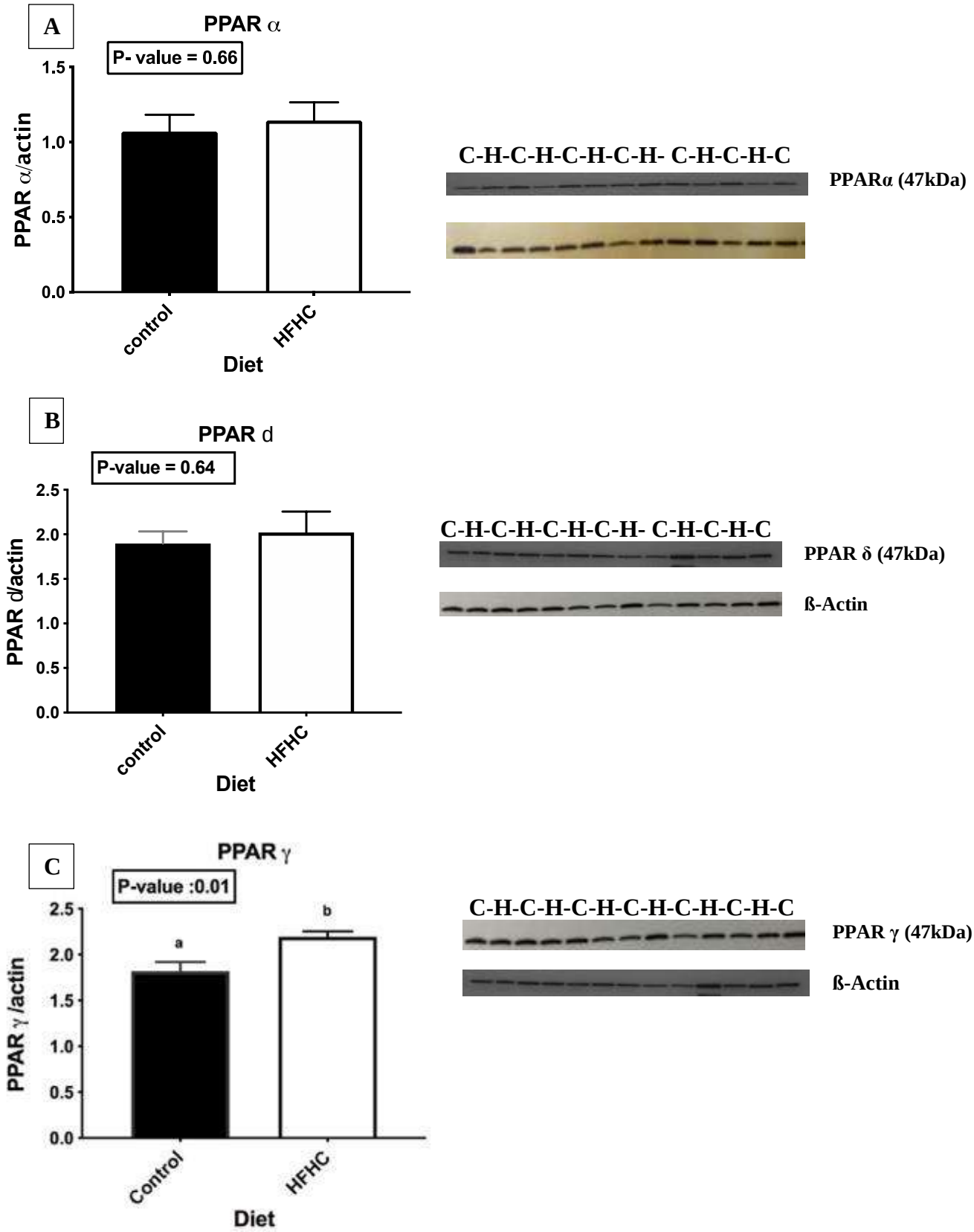

Figure 3. 1 The effect of exposure to a HFHC diet on protein expression of Peroxisome Proliferator Activated Receptor (PPARs): (A) PPAR-α, (B) PPAR-δ, (C) PPAR-γ in the term labour placenta. C: Control group, H : HFHC group. Sample size control groups were n=7, and HFHC group was n= 6. Values are means±SEM. Statistical analysis was Independent T-test with significant differences at the P≤0.05 level

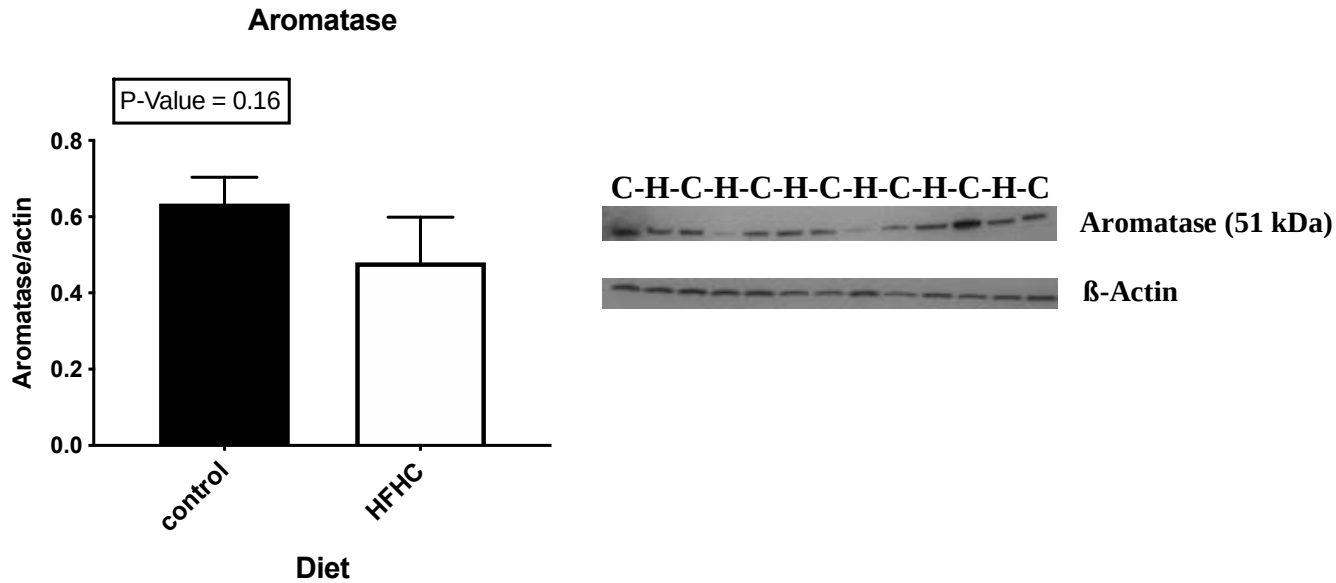

Figure 3.2 The effect of the HFHC diet on the protein expression level of aromatase. The sample size for controls was n=7, and HFHC group n= 6. C: Control group, H : HFHC group Values are means±SEM. Statistical analysis was by Independent T-test at the P≤0.05 level.

3.4 Discussion

This study stated that more than 50% of placental fatty acid in control and HFHC group was saturated fatty acid. Fonseca et al., (2018) also identified that saturated fatty acid is the highest proportion of fatty acid in placenta.

The key finding from the current experiment that exposure to the HFHC diet significantly alters the fatty acid profile of the placenta. The fatty acid profile in the placenta from HFHC fed rat was characterised by significant lower in saturated fatty acid, but increases in myristic acid, tridecylic acid, arachidic acid and DGLA. The potential reason of lower proportion of saturated fatty acid in HFHC group compared than control group was that pregnant rat that fed with HFHC group might transferred more saturated fatty acid to the foetus compared with control diet group as mentioned by Hirschmugl et al., (2021) that direct transfer of saturated free fatty acid from mother to foetus is higher in obese compared with normal women. Furthermore, increased number

of myristic acid in HFHC diet group was same with another study conducted by Cerf et al., (2016) that shows significantly increase of rat placental neutral lipid and phospholipid myristic acid concentration in high fat fed rat during last trimester in comparison with control diet.

DGLA significantly increased in HFHC group and it might associate with ELOVL 5 as reported by Wang et al., (2008) that identifies elevating Elovl5 activity increase hepatic and plasma levels of DGLA but decrease hepatic AA and DHA content. The same results figure in this study which the number of DGLA in HFHC group increased significantly but AA was not different between HFHC group control group. Moreover, Lee et al., (2016) reported that DGLA and Eicosatetraenoic acid (ETA) compete to use delta-5 Desaturase to produce longer chain fatty acid such as Arachidonic acid (AA) and Eicosapentaenoic acid (EPA).It was identified that arachidonic acid levels were similar between the control and HFHC fed rats, although the level of DGLA was significantly higher in the HFHC fed rats. These probably DGLA doesn't have sufficient delta-5 Desaturase to convert into high amount AA which make level AA in HFHC was not significant different compare with control group.

The exciting evidence within this study is that there were no differences in the omega-3 and omega-9 fatty acids between rats exposed to a HFHC or control diet, but DGLA omega-6 was significantly higher in HFHC group compared with control group. Cross sectionally analysis conducted by Tsurutani et al., (2018) reported that increased serum DGLA level has a stronger association with obesity, body fat accumulation and insulin resistance and its related to the downregulated activity of delta5-desaturase, an enzymes that converting DGLA to AA in patient with type-2 diabetes mellitus. Moreover, involvement of several nuclear transcription factors, including PPARs might responsible to fatty acid uptake increase several fatty acid proportion as mentioned by

Calabuig-Navarro et al., (2016) that reported a negative correlation between PPARγ with the n-3:n-6 ratio in the placenta of obese women supplemented with omega-3.

Furthermore, increased proportion of DGLA in the HFHC group might contribute to lead prolonged labour through proinflammation cytokine inhibition. This theory supported by Dooper et al., (2003) that identified 100 µM DGLA supplementation into isolated human peripheral blood mononuclear cells for 48 hours significantly decrease kinetics of of IL-10 and TNFα level up to 60%. Many studies provide evidence that TNF α can increase myometrium contraction through increase production of prostaglandin tpe-2 (Sivarajasingam et al., 2016). Further evidence also proved that adding 20 µM DGLA in the IL-1 stimulated synovial cells decrease concentration of PGE_2 up to 70% (Baker et al., 1989) where PGE2 has been identified to inhibit myometrium contraction.

Another potential mechanism related prolonged and dysfunctional labour could be associated with increased protein expression of PPAR γ since this study provides evidence that the HFHC diet significantly increased PPAR γ expression in the placenta. This result is supported by Navarro et al., (2017) who identified that increasing levels of PPAR γ protein expression in the trophoblast cells of placentas from obese pregnant women at term prior to caesarean section. Furthermore, the high proportion of myristic acid in the HFHC group might also potentially increase protein expression of PPARγ. These evidence supported by Sheng et al (2014) that identified supplementation myristic acid in the intramuscular adipocytes cells increase mRNA and protein expression of PPARγ. This result suggests that a high fat diet may potentially cause prolonged and dysfunctional labour via PPARγ activiation since it has been reported in many studies that PPARγ maintains uterine quiescence during pregnancy in human and animal (Froment et al., 2006; MacLaren et al., 2006; Pascual et al., 2005; Schaiff et al., 2006).

The potential mechanism of PPARγ prolonging labour could be that high PPAR γ protein expression suppresses pro-inflammatiatory molecules such as NF-Kb, IL-β, TNFα, IL-6, IL-8 resulting in inhibition of uterotonic prostaglandins (Borel et al., 2008). Many studies confirm that up regulation of PPARγ decreases proinflammatory cytokines such as TNF, IL6, IL8 and NfKB gene expression and resultant prostaglandin release from gestational tissues including the placenta (Lappas et al., 2002). However, future study needed to prove the association of PPARγ in cytokine expression in maternal obesity.

Another plausible mechanism of PPAR γ increase the risk of delayed labour with maternal obesity is PPAR γ enhance VEGF in human macrophage model and vascular smooth muscle cell where many studies identified that VEGF has important role to increase progesterone secretion in plasma (Bamba et al., 2000; Fraser and Wulff, 2003; Froment et al., 2006; Yamakawa et al., 2000). Another study supports this theory that mentioned VEGF expression in the corpus luteum associate with increased plasma progesterone and suppressed myometrial contraction in mice (Wada et al., 2013). From the explanation above, it suggests that increased protein expression of PPAR γ in the placenta HFHC fed rat potential to increase progesterone production through VEGF at term labour rat and this might become one of mechanism behind prolonged labour in maternal obesity. However, future studies need to be carried out to confirm the expression of VEGF after HFHC supplementation during pregnancy. The potential mechanism that high fat diet decreased myometrium contraction through increased of placenta PPAR γ protein could be seen in **figure 3.3.**

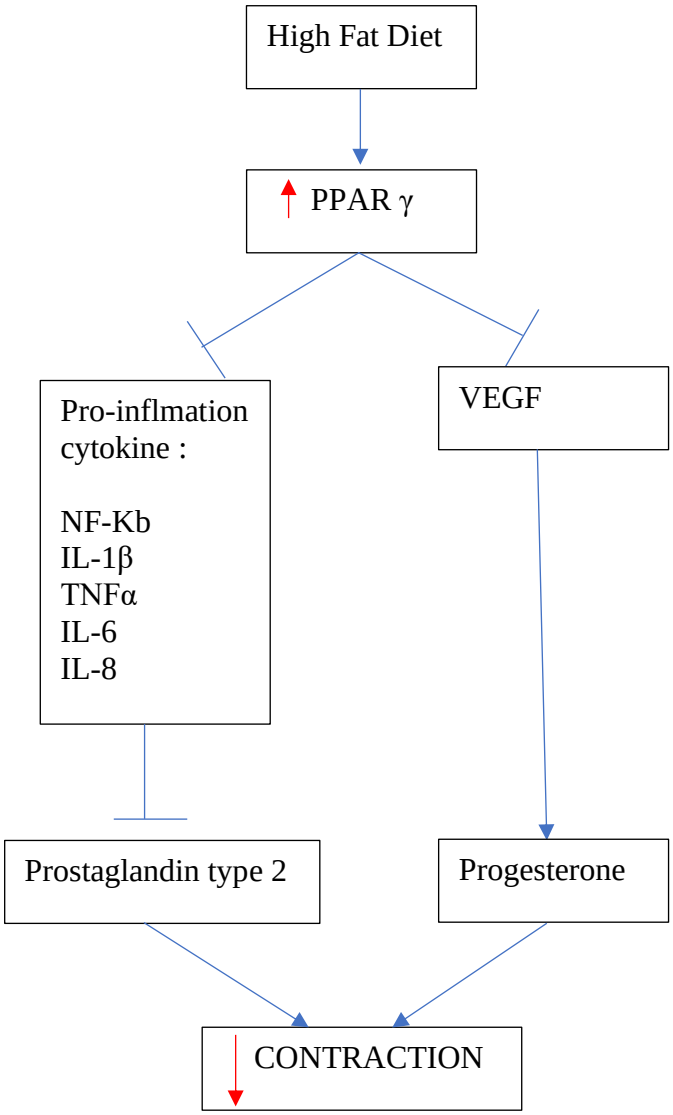

Figure 3. 3 The effect of high fat diet on peroxisome proliferator activated receptor (PPAR γ) in the placenta and the potential mechanism behind PPAR γ increasing the risk of delayed labour in pregnant rats through proinflammatory cytokine suppression that potential to decrease prostaglandin type-2 synthesis and contraction during labour. NFkB : Nuclear Factor Kappa B, , IL-8 : Interleukin-8, IL-10 : Interleukin 10, IL-6 : Interleukin-6, IL1β : Interleukin-1 β, VEGF : Vascular Endhotelial Growth Factor.

During parturition, progesterone increases slowly, while estrogen increase dramatically in human parturition. Aromatase is a key enzyme in the synthesis of estrogen by converting androstenedione to estrone (Froment et al., 2006). Previous studies have shown that downregulation of aromatase is associated with PPARγ activities in human ovarian cells and in murine mammary glands (Fan et al., 2005; Margalit et al., 2012). However, the present study confirmed that PPAR γ was not decreased aromatase

protein expression of the placenta. PPAR γ might not downregulate COX-2 and PGE_2 signalling. The established study illustrated that plasma PGE_2 concentration and uterus COX-2 protein expression between HFHC and control group was not significantly different, which suggested that PPAR γ might not affect COX-2 and PGE_2 signalling. Therefore, increased protein expression of PPAR γ would not affect aromatase in the placenta. This theory is in line with Subbaramaiah and colleagues (2012) and Cruz et al., (2005), which reports that PPARγ inhibits aromatase activity through decreased level of protein COX-2 and PGE_2 in human and mice preadipocyte mammary gland cells. Furthermore, Cruz., (2005) also stated the positive relationship between COX-2 and aromatase gene expression in breast cancer specimen.

Aromatase is not the only enzyme that regulates estrogen production which could be affected by PPAR γ. The authors speculate that PPARγ might inhibit another enzyme that acts as an oestrogen precursor such as Cyp17 (P450c17α) and 17α-hydroxylase. A previous study confirmed that the activity of 17α-hydroxylase increases by 600-fold at the end of parturition in ewes (Mason et al., 1989; Tsumagari et al., 1993). In addition a study also mentioned that PPAR γ impairs the ability of Cyp17 (P450c17α) to synthesise androstenedione in ovarian theca cells (Schoppee et al., 2002). Decreasing androstenedione might decrease synthesis of oestrogen in the placenta. Therefore, future study might be needed to prove whether increased protein expression PPAR γ will decrease Cyp17 (P450c17α) and 17α-hydroxylase enzymes and inhibit oestrogen synthesis in the placenta.

3.5 The strength and limitation study

The current study has identified that a high fat diet modified fatty acid composition of the placenta and a potential mechanism leading to prolonged labour through increased PPARγ protein expression. However, this study has some limitation that should be note for future study. The first is, this study only measured the percentage of fatty acid in maternal placenta that might not figure the absolute concentration of fatty acid, therefore, future study needed for Investigate the concentration of placenta fatty acid profile in phospholipid and neutral lipid might comprehensively assess the fatty acid content placentae in both normal and obese pregnancy.

Another limitation on this study is PUFA was not detected in high percentage. This might because some of reasons such as some unsaturated FAs might lose through oxidation when sample was allowed to dry out during analysis. The other potential reason is there might reagents were impure and this could have introduced other compounds then detected by the GCFID. The unknown compound name that detected in placenta after confirmed with GC-MS analysis was ethyl 2, Lactone, Napthuric acid and octadenoic acid. The unknown compound which detected in GC FID and GC-MS might have a better affinity with the detector and the peaks will appear larger than the actual concentration would be in relation to the other peaks on the chromatogram.

Furthermore, this current study did not investigate the effect of increased placental PPARγ in the synthesis of proinflammatory cytokines such as NfKb which affect COX-2 and aromatase expression as Fan et al., (2005) identified that PPARγ activation can inhibit aromatase transcription via NfKb in cultured human ovarian granulosa cells. Therefore, it would be useful for future research to analyse NfKb protein expression in placental tissue or Nfkb activity placental of obese rats in greater detail to help determine the pathway behind prolonged and dysfunctional labour with maternal

obesity. It is important to note that although this study identified that aromatase was not affected by PPARγ expression in the placenta. The unsignificant different of aromatase expression between control and HFHC group might due to power limitation, since control group has 7 samples, and HFHC only has 6 samples. Increased number of sample potential affect expression between HFHC and control group. Furthermore, there are other potential enzymes like Cyp17 and 17α-hydroxylase that regulate oestrogen synthesis that could potentially be affected by PPAR γ which again might be useful to analyse in the future. Identification of the relationship between PPAR γ and Cyp17 and 17α-hydroxylase expression in the placenta can help to identify whether the mechanism behind prolonged and dysfunctional labour with maternal obesity is through decreasing oestrogen but increasing progesterone production during parturition as reported by Crew et al., (2016) that identified an increase in plasma progesterone concentrations in term pregnant rats after high fat supplementation during pregnancy.

4. The Effects of Increasing Omega-3 PUFA Status on Uterine Expression of CAPs and PPARs

4.1 Introduction

The result of the previous chapter in this thesis provide evidence that rats exposed to a High Fat and High Cholesterol diet (HFHC) prior to and during pregnancy decreased omega-3 and omega-6 PUFA proportions in both the plasma and liver, whereas in the uterus, only omega-3 decreased. Moreover, switching diet from HFHC to control diet at conception improved the omega-3 and omega-6 status in plasma and liver, however interestingly, only omega-3 proportion significantly increased in the uterine horn. There is some evidences that chronic exposure to a HFHC diet adversely affected expression of key contractile association protein (CAPs) including Cav-1, CX-43, PCX-43, COX-2 and OXTR, thus suggesting lipid might play a role in regulating the expression of CAPs and myometrial contractile activity. (Miklos and Frank, 2000; Muir et al., 2016; Patterson et al., 2012).

Some studies report the effect of omega-3 and omega-6 PUFA supplementation on the timing and length of gestation. Olsen et al., (1992) and Smuts et al., (2003) mentioned that omega-3 PUFA supplementation during the last trimester of pregnancy in women significantly decreased the risk of preterm birth and caesarean delivery. On the other hand, Elmes et al., (2005) reported that raising intake of omega-6 PUFA Linoleic acid (LA) in the diet from 100 days' gestation increase maternal and foetal circulation of prostaglandin type-2 production during labour that increases the risk of preterm labour in the ewes.

Omega-3 and omega-6 PUFAs compete with each other to use the same enzymes to desaturation and elongation, producing prostaglandin type 2 in which increased $PGF_{2\alpha}$ might result in higher risk for preterm labour (Yashiro et al., 2016). The study conducted by Torres et al., (2006) identified that increasing the omega-6:

omega-3 ratio increased $PGF_{2\alpha}$ production in bovine endometrial cells. The omega-6 increased 2 series prostaglandin through the precursor arachidonic acid which is a substrate for COX-2, the enzymes that synthesis the bioactive 2 series prostaglandins (Ringbom et al; 2001; Smith et al., 1991). COX-2 increases $PGF_{2\alpha}$ and stimulates uterine contractions during labour (Schuler et al., 2006). Another study support this theory that stated inhibition of uterus mRNA COX-2 prevent inflammation that mediate preterm labour in the mouse (Gross et al., 2000). However, the omega-3 LCPUFA DHA inhibit COX-2 protein expression through PKC activation in human vein endothelial cells (Massaro et al., 2006).

The ability of two series prostaglandin to stimulate contractions is also heightened by an increase of uterine, decidua and ovary concentration of OXTR receptor in human and animals study (Blanks et al., 2003; Soloff et al., 2000). The activation of OXTR promotes myometrial contractions through Ca^{2+}-calmodulin-MLCK pathways (Arrowsmith and Wray, 2014). Kim et al., (2012) found a significant decrease of oxytocin-induced Ca^{2+} intracellular release after 48 hours treatment with 10-100 µM DHA or LA in pregnant human myometrial smooth muscle cells. However, Torres et al., (2006) reported that increasing the omega-6: omega-3 ratio increase concentration of $PGF_{2\alpha}$ in phorbol ester-stimulated bovine endometrium cells. On the other hand, the same study also stated that 24 hours EPA preincubation reduces $PGF_{2\alpha}$ response to phorbol ester-stimulated bovine endometrium cells. Cheng et al., (2010) provided evidence that ALA induces a small increase in PGE_2 production whilst the longest chain omega-3 PUFA DHA inhibited PGE_2 synthesis in amnion cells *in-vitro*. The fact that omega-3 fatty acids have been shown experimentally to decrease prostaglandin type-2 synthesis, could be associated with a decrease in COX-2 and OXTR protein expression in the pregnant uterus rat tissue which might alter labour

outcome. The experiment conducted by Ma et al., (2000) supported the theory that intravenous administration of omega-3 significantly inhibits mRNA COX-2 and OXTR in the ewes myometrium during labour.

The other potential mechanism through which omega-3 PUFAs might alter the outcome of labour might be via alteration of PPAR activity. PPAR can suppress COX-2 expression through pro-inflammation inhibition such as Nf-Kb and IL-β (Ackerman et al., 2005a; Dong et al., 2013; MacLaren et al., 2006; Wang et al., 2016). Research provides evidence that PPAR γ protein expression declines once active labour commences, and coincides with an increasing level of COX-2 expression in fetal membranes (Dunn-Albanese et al., 2004). The omega-3 PUFAs, especially DHA and EPA are natural ligands of PPARα/γ/δ in endothelial cells (Grygiel-Górniak, 2014b). EPA and AA are efficient activators of PPARα and PPARδ, but display only little activation on PPARγ (Forman 1997). Further evidence supporting omega-3 consumption increases mRNA expression level of PPARγ-target genes such as FATP, IL-1 and CX-43 in cardiac tissue and cultured neonatal cardiomyocytes rat (Shysh et al., 2006). Dietary supplementation of omega-3 PUFAs EPA and DHA have been reported to increase PPARα activity and inhibit Nf-Kb signalling in rats following hepatic ischemia reperfusion injury (Zuniga et al., 2011). This finding suggests that increasing omega-3 PUFA in the diet could suppress COX-2 expression through increased activity of PPARs, and could potentially be the same mechanism in the uterus and cause relaxation of the myometrium during labour.

No study to date has investigated the effect of dietary omega-3 PUFA on expression of the CAPs CX-43, PCX-43 and Cav-1 within the term uterus. The evidence stated that omega-3 PUFAs EPA and DHA modifies the location and function of Connexin and Cav-1 protein in cell membranes including caveolae and lipid

rafts (Puebla et al., 2017). Cav-1 interact with CX-43, whereas Cav-1 regulate gap junction intercellular communication (GJIC) and the presence of CX-43 in lipid raft domains may contribute to the mechanism modulating GJIC (Li et al., 2007; Schubert, 2002). The review study suggest that expression of Cav-1 has found in caveolae of smooth muscles and the Cav-1 scaffolding domain is a region crucial for regulatory interaction with diverse signal transducing including inhibition translocation of RhoA and ROK that involved in contractile activation (Taggart, 2001). Moreover, the evidence shows that Cav-1 knockout from male mouse smooth muscle intestine decrease frequency of contraction per minutes and amplitude of contraction *in vivo* (Daniel et al., 2009). Uterus is an organ that consist smooth muscle, therefore, the same mechanism might occur in the uterus.

Another CAP, CX-43 also essential to synchronize uterus contraction by regulating intercellular communication through gap junction plaques (Lampe and Lau, 2004). CX-43 increase in size and abundance during parturition (Kilarski et al., 2001), and ablation of CX-43 in the uterus, causing prolonged labour in the mouse (Doring et al., 2006). Interestingly, it has been report that increasing exposure to omega-3 PUFAs increase the abundance of immune-labelling of CX-43 and enhance protein and mRNA CX-43 in the diabetic rat heart. Another evidence from the same study also stated that omega-3 could repair disorganization and reduce the abnormal distribution of CX-43 in the diabetic rat heart (Radosinska et al., 2015). The fact that omega-3 PUFA has been identified to increase CX-43 abundance in the diabetic rat heart might suggest that the same mechanism to be true in the uterus and potential to affect myometrium contraction during labour.

4.1.1 Objective and Hypothesis

The previous study identified that only total omega-3 PUFA decreased in the uterus after exposure to a HFHC diet during pregnancy. In addition the established study conducted by our research group showed that the HFHC diet alters protein expression of CAPs in the uterus. Therefore, the aim of this study was to investigate the effect of different ratios of LA:ALA fed at a low or high level of fat on the fatty acid compostion of the plasma, liver and uterus. Another objective of the study was to determine whether improving omega-3 PUFA status by increasing consumption of alpha linolenic acid (ALA) would induce alterations in protein expression of key CAPs and PPARs in the pregnant term rat uterus. LA and ALA diet were chosen in this study because LA and ALA responsible for their respective long-chain derivatives, and for incorporation into cell membranes. Omega-3 and omega-6 may potentially increase membrane fluidity and affect signalling including CAPs and PPARs. Furthermore, omega-3 FAs such as EPA, DHA and omega-6 FAs are natural ligands for PPAR activation. The study hypothesis was that increasing omega-3 PUFA consumption before and during pregnancy will increase the protein expression of CAPs and alter PPAR protein expression.

4.2 Materials and Methods

4.2.1 Animal Study

Twenty weanling Wistar rats, approximately 60 grams in weight, were used in these experiments. All rats were allowed to acclimatise for one week in the animal unit before being fed either 1) a high omega-6 diet (9:1 ratio, 18% fat, n=4), 2) a high omega-6 diet (9:1 ratio, 36% fat, n=4), 3) a high omega-3 diet (1:1.5 ratio, 18% fat, n=4), and 4) a high omega-3 diet (1:1.5 ratio, 36% fat, n=5). The ratio represented the comparison between linoleic acid and alpha-linolenic acid (LA: ALA). The composition of LA and

ALA was relevant to the comparison of LA and ALA in the western diet over the past decade (Blasbalg et al., 2011). The composition of the diet can be seen in Table 4.1, while the composition of the fatty acids within the diet are shown in Table.4.2. After four weeks feeding, each rat was mated, and the appearance of a semen plug was confirmed as day 0 of pregnancy. Pregnant rats were then housed and maintained on their respective diet throughout pregnancy. At day 20 of pregnancy, the rat dams were euthanised by CO_2 asphyxiation and the uterus collected and snap-frozen in liquid nitrogen before being stored at -80°C for western blot analysis.

Component	Amount (%)			
	High LA Low Total Fat (9:1 18%)	**High LA High Total Fat (9:1 36%)**	**High ALA Low Total Fat (1:1.5 18%)**	**High ALA High Total Fat (1:1.5 36%)**
HiCasein	16.0	16.0	16.0	16.0
Cornflour	31.8	20.0	31.8	20.0
Sucrose	16.0	10.0	16.0	10.0
Cellulose	15.6	15.6	15.6	15.6
Mineral Mix	1.8	1.8	1.8	1.8
Vitamin Mix	0.4	0.4	0.4	0.4
Choline Chloride	0.2	0.2	0.2	0.2
Methionine	0.4	0.4	0.4	0.4
Flaxseed Oil	0.9	1.7	4.2	8.5
Sunflower Oil	6.6	13.1	0.8	1.7
Macadamia Oil	6.9	13.8	9.5	19.0
Coconut Oil	3.4	6.9	3.2	6.3
Total Fat	**17.8**	**35.6**	**17.8**	**35.5**

Table 4. 1 Fat composition (%) of each component diet in high LA and high ALA group. The 17.8% of total fat represented low fat diet, while 35.6% represented the high fat diet.

Fatty Acid Composition in Each Group Diet	Amount (% of total lipids) High LA Low Fat (9:1 18%)	High LA High Fat (9:1 36%)	High ALA Low Fat (1:1.5 18%)	High ALA High Fat (1:1.5 36%)
14:0	4.2	4.2	4.1	3.7
15:0	0.0	0.0	0.0	0.0
16:0	10.5	10.3	10.8	10.5
17:0	0.1	0.1	0.1	0.1
18:0	4.1	4.0	4.3	4.0
20:0	0.8	0.8	1.0	1.0
22:0	0.7	0.7	0.5	0.5
24:0	0.3	0.3	0.3	0.2
Total Saturates	20.7	20.3	21.1	20.0
t18:1n-9	0.0	0.1	0.1	0.1
t18:1n-7	0.0	-	0.0	-
Total Trans	0.1	0.1	0.1	0.1
16:1n-7	3.9	4.0	5.2	5.5
18:1n-9	42.9	43.1	45.7	47.1
18:1n-7	1.3	1.3	1.5	1.4
20:1n-9	0.7	0.7	0.8	0.9
22:1n-9	0.1	0.1	0.1	0.1
24:1	-	0.0	-	0.0
Total Monounsaturates	48.8	49.0	53.3	54.9
18:3n-3	**3.1**	**3.2**	**14.4**	**14.4**
Total Omega-3	3.1	3.2	14.4	14.4
18:2n-6	**27.3**	**27.3**	**11.1**	**10.5**
20:2n-6	0.0	0.0	0.0	0.0
20:3n-6	0.0	0.0	0.0	-
Total Omega-6	27.3	27.4	11.1	10.5
Total Polyunsaturates	30.4	30.6	25.5	25.0

Table 4. 2 Fatty acid composition in each group diet; high LA followed with high fat diet (9:1 36%), high LA followed with low fat diet (9:1 18%), high ALA followed with high fat diet (1:1.5 36%), high LA followed with low fat diet (1:1.5 18%).

4.2.2 Fatty acid analysis of the uterus (Lipid extraction, Fatty Acid Methyl Ester (FAME) And Gas Chromatography analysis)

The protocol for fatty acid analysis of the uterus was identical to placental fatty acid analysis in **Chapter 3**. For lipid extraction Fatty Acid Methyl Ester (FAME) and gas chromatography procedures please refer to sections **3.2.3 to 3.2.4** of the thesis.

4.2.3 CAPs and PPARs analysis

4.2.3.1 Tissue preparation

Uterine rat tissue weighing approximately 300 mg was crushed with pestle and mortar under liquid nitrogen and then homogenised for 30 seconds in the presence of 2 ml ice-cold extraction buffer (32.5 mM Tris/HCL, 3.08mM EDTA, PH 7.5) and 10 µl protease inhibitor cocktail III (calbiochem, San Diego,CA, USA). Samples were then centrifuged at 13000g for COX--2 and 3500 for CX-43, Cav-1, OXTR and PCX-43 and PPARs for 10 minutes at 4°C to isolate the supernatant. Supernatant was divided into two tubes, one tube for protein assay and the other tube containing the remaining supernatant was diluted with the same amount of 2x sodium dodecyl sulphate mix (SDS). The 2XSDS made from 1.25ml 1M Tris pH 6.8, 4 ml 10% (w/v) SDS, 2 ml glycerol, 1ml 1M DTT (0.154g/ml) or 1 ml beta-mercaptoethanol, then add 1 to 3 mg bromophenol blue and made up to 10 ml with distilled water then kept in -20°C until used for western blot analysis.

4.2.3.2 Protein assay and western blot analysis

The protein assay methods are referred to in Chapter 3 section 3.3.5, while western blot analysis section 4.3.6. Moreover, western blot analysis was used to identify the CAPS Cav-1, CX-43, COX-2, Pcx-43, OXTR, and PPARs. The molecular weight of the CAP's and PPARs proteins are described in Table 4.3.

Protein	Molecular weight (Kda)
Cav-1	22
CX-43	37
COX-2	72
Pcx-43	37
OXTR	60
PPARs	47

Table 4. 3 Molecular weight protein of interest

The membrane was blotted overnight at 4°C with conjugated anti-mouse IgG primary antibody (Cav-1 1:1000, Abcam UK), CX-43, 1:5000 Abcam, UK), (OXTR 1:10,000, abcam UK), COX-2 (1:3000,, Abcam UK), (PCX-43 1:2000, Abcam, UK), (PPARα,1:2000, PPARδ PPAR γ 1:1000, Abcam, UK) . After that, 5 minutes X 12 wash cycle with 1XTBST solution to reduce unspecific binding of the primary antibody. Following this wash cycle, the membranes were blotted with conjugated anti mouse IgG secondary antibody for Cav-1, Cx43, COX-2 and OXTR, PPAR α, PPAR δ, PPAR γ were 1:4000, 1:25,000, 1:20,000 and 1:80,000, 1: 30,000, 1;40.000, 1:40.000 respectively) for 1 hour at room temperature. Again, a 5 minutes X 12 wash cycle followed to reduce unspecific binding of secondary antibody and reduce background exposure. The membrane was then incubated with ECL detection reagent (GE Healthcare UK Ltd). for 5 minutes at room temperature to initiate the chemiluminescent reaction. The membrane was then exposed to photogenic film (GE Healthcare UK Ltd) under red light to capture the protons emitted from chemiluminescent reaction. After film was developed and fixed, the protein expression was quantified using a Bio-Rad Gel-doc analysis (Bio-Rad, USA). β-actin was used as the housekeeping protein (Sigma Aldrich, UK, 42KDa, 1:10,000) with anti mouse secondary antibody IgG horseradish peroxide (1:40,000) (GE UK Healthcare Ld, UK). For β-actin analysis, membranes stripped with Restore PLUS Western Blot Stripping Buffer (Thermo-Fisher Scientific UK) for 30 minutes at room temperature post detection of CAPs and PPARs

to prepare the membrane for blotting of the housekeeping protein following the same method stated previously for western blotting.

4.2.3. Data analysis and Statistic Analysis

Statistical analysis was carried out using SPSS version 24; SPSS inc, Chicago, IL, USA and expressed as the mean values ± standard mean error (SEM). The homogeneity of the data was assessed and if not normally distributed, the data was transformed log 10 to achieve a normal distribution. The Two-way ANOVA analysis was used to determine the effect of diet, level of fat, and interaction between diet and fat of the plasma fatty acids profiles (saturated, monounsaturated, total omega-3, EPA, ALA, DPA, DHA, AA and total omega-6) and uterine fatty acid profile , uterine CAPs and PPARs expression. The Fisher's LSD tests was used to determine multiple comparisons and significance differences were set at the level of $P<0.05$. Meanwhile, the effect of diet, level of fat and interaction between diet and fat with plasma LA proportion was analysed with non-parametric Friedman test followed with Bonferroni test for analysed multiple comparisons at level of $P<0.05$.

4.3 Result

4.3.1 The effects of dietary omega-3 PUFA consumption on the fatty acid profile of plasma and uterus

The differences in plasma fatty acid proportion (%) between the high LA and ALA diet with different fat levels is presented in Table 4.4, while fatty acid proportion (%) in the uterus can be seen in Table 4.5. The (1:1.5 36%) group had the lowest saturated fatty acid compared with all groups ($P<0.05$). Exposure to the high omega-3 PUFA (ALA) diet significantly increased total plasma concentrations of omega-3,

ALA, EPA and DPA with P-value < 0.01. Conversely, the plasma level of total omega-6, LA and AA was lower in the high ALA (1:1.5) group when compared to the high LA omega-6 diet (9:1) group (P<0.01). Statistical analysis identified that the level of fat fed had an impact on total plasma saturated fatty acids (P= 0.04), LA, EPA (<0.01), and ALA (P= 0.04). There was also a significant interaction between fat and diet in plasma LA (<0.01) and DPA (0.04). The level of plasma DPA in the (1:1.5) group was 2 until 3 fold higher than (9:1) groups.

Chronic exposure of varying ratios of LA and ALA and level of fat fed had little effect on the fatty acid profile of the uterus. There was no correlation of saturated fatty acid between all group. Meanwhile, the proportion of AA and EPA was significantly different with the highest percentage of AA was in the (9:1 18%) with more than 20 fold higher compared than all group. Moreover, high exposure to high ALA diet followed with low fat significantly increased EPA proportion in the uterus with proportion 0.20%. The proportion of (1:1.5 36%) group was significantly lower than the (1:1.5 18%) group. This suggest that fat diet potential to affect EPA and AA proportion in the uterus.

Plasma	High ALA High Fat (1:1.5 36%) (n= 4) Mean±SEM (%)	High LA high Fat (9:1 36%) (n= 3) Mean±SEM (%)	High ALA Low Fat 1:1.5 18% (n= 4) Mean±SEM (%)	High LA Low Fat (9:1 18%) (n= 3) Mean±SEM (%)	P-value (Fat)	P-value (Diet)	P-value Diet*Fat
Total Saturated	35.05±1.04[a]	39.81±2.67[b]	39.56±0.19[c]	40.92±0.67[b]	0.04	0.05	0.23
Total Monounsaturated	29.49±1.67[a]	18.81±3.09[b]	24.79±1.11[a]	19.91±0.79[b]	0.34	0.01	0.14
omega-6							
18:2n-6/ LA	13.33±0.17[a]	15.84±2.28[b]	11.96±0.37[a]	14.51±0.73[b]	<0.01	<0.01	<0.01
20:4n-6/ AA	9.36±0.70[a]	18.76±1.31[b]	10.95±0.37[a]	18.77±0.49[b]	0.47	<0.01	0.23
Total omega-6	23.44±0.79[a]	37.12±0.57[b]	23.69±1.06[a]	35.13±0.75[b]	0.33	<0.01	0.22
omega-3							
18:3n-3/ ALA	5.05±0.63[a]	0.60±0.23[b]	3.21±0.31[c]	0.49±0.03[b]	0.04	<0.01	0.07
20:5n-3/ EPA	2.95±0.29[a]	0.17±0.01[b]	4.11±0.29[c]	0.3±0.01[b]	<0.01	<0.01	0.15
22:5n-3/ DPA	1.51±0.14[a]	0.77±0.08[b]	2.05±0.14[c]	0.75±0.01[b]	0.06	<0.01	0.04
22:6n-3/ DHA	2.24±0.14[a]	2.38±0.49[b]	2.27±0.85[a]	2.15±0.15[b]	0.93	0.55	0.46
Total omega-3	11.74±0.46[a]	3.93±0.15[b]	11.64±0.23[a]	3.69±0.11[b]	0.45	<0.01	0.77

Table 4. 4 The fatty acid profile in the plasma after varying ratio LA: ALA followed with different level of fat diet during pregnancy. The group of diet were high ALA and high fat diet (1:1.5 36%), high ALA and low fat diet (1:1.5 18%), high LA and high fat diet (9:1 36%), high LA and low fat diet (9:1 18%). LA: Linoleic Acid, AA : Arachidonic acid, ALA : Alpha Linolenic acid, EPA : Eicosapentaenoic acid DPA : Decosapentaenoic acid, DHA : Decosahexaenoic acid. Values are means±SEM. Statistic analysis was used two-way ANOVA for saturated fatty acid, monounsaturated, total omega-3, EPA, ALA, DPA, DHA, AA and total omega-6. Fisher's LSD tests was used to determine multiple comparisons. Meanwhile LA was analysed with non parametric test Friedman test followed with Bonferroni test for analysed multiple comparisons. The different superscript letters signify significant differences at the P<0.

Uterus	High ALA Low Fat 1:1.5 36% (n= 4) Mean±SEM	High LA high Fat 9:1 36% (n=3) Mean±SEM	High ALA Low Fat 1:1.5 18% (n= 4) Mean±SEM	High LA Low Fat 9:1 18% (n=3) Mean±SEM	P-value (Fat)	P-value (FA)	P-Value FA*FAT
Total Saturated	63.16±8.16	66.33±8.16	67.66±2.98	57.88±12.47	0.80	0.67	0.41
C4:0/ Butyric acid	0.02±0.02	0.04±0.02	0.02±0.02	0.10±0.08	0.43	0.18	0.62
C6:0/ Caproic acid	0.48±0.24	0.05±0.01	0.22±0.22	0.20±0.17	0.80	0.27	0.32
C8:0/ Caprylic acid	0.04±0.03	0.08±0.02	0.04±0.0 2	0.04±0.03	0.38	0.45	0.38
C10:0/ Capric acid	0.59±0.37	0.333±0.05	0.03±0.02	0.16±0.08	0.38	0.44	0.81
C12:0/ Lauric acid	12.32±4.62	3.22±1.03	3.67±1.86	3.41±0.10	0.18	0.14	0.17
C13:0/ Tridecylic acid	0.015±0	0.03±0.02	0.02±0.01	0.02±0.04	0.18	0.14	0.17
C14:0/ Myristic acid	5.83±1.56	2.96±0.33	3.11±0.85	3.18±0.46	0.25	0.20	0.19
C15:0/ Pentadecylic acid	0.36±0.15	0.68±0.17	0.65±0.15	0.47±0.27	0.95	0.58	0.21
C16:0/ Palmitic acid	29.08±3.77	28.13±1.27	32.39±1.53	26.72±4.39	0.66	0.20	1
C17:0/ Margaric acid	$0.20±0.09^{a}$	$0.81±0.24^{bc}$	$0.53±0.04^{b}$	$0.55±0.36^{b}$	0.52	0.22	**0.03**
C18:0/ Stearic acid	20.81±3.02	27.46±4.96	24.29±3.79	21.03±8.60	0.60	0.26	0.09
C20:0/ Arachidic acid	0.21±0.12	0.60±0.12	0.45±0.20	0.42±0.24	0.85	0.28	0.22
C21:0/ Heneicosylic acid	0.02±0.02	0.37±0.32	0.038±0.01	0.08±0.05	070	0.21	0.07
C22:0/ Bahenic acid	0.16±0.09	0.65±0.08	0.32±0.15	0.39±0.22	0.72	0.06	0.12
C23:0/ Tricosylic acid	0.10±0.06	0.20±0.14	0.04±0.01	0.16±0.11	0.36	0.36	0.13
C2:0/ Lignoceric acid	0.96±0.60	0.66±0.15	1.55±1.26	0.94±0.36	0.54	0.52	0.83
Total Monounsaturated	0.59±0.22	1.41±5.09	0.85±0.34	0.70±0.36	0.56	0.39	0.22
C14:1/myristic acid	0.07±0.06	0.04±0.00	0.08±0.0	0.09±0.07	0.04	0.30	0.28
C15:1/ Pentadecenoic acid	0.15±0.16	0.02±0.01	0.02±0.010	0.05±0.02	0.66	0.31	0.63
16:1n-7/ Palmitic acid	0.18±0.18	0.21±0.07	0.08±0.04	0.15±0.09	0.27	0.50	0.75
C17:1/ Heptadecenoic acid	0.12±0.14	0.99±0.62	0.71±0.39	0.26±0.13	0.89	0.55	0.40
C24:1/ Nervonic acid	0.08±0.06	0.14±0.10	0.01±0.01	0.15±0.14	0.12	1	0.30
Total Omega 9	1.14±0.54	0.79±0.01	1.76±1.5	0.72±0.43	0.74	0.41	0.68
18:1n-9/ OA	1.11±0.53	0.68±0.02	0.35±0.29	0.58±0.32	0.40	0.70	1
22:1n-9/ Erucic acid	0.01±0.02	0.10±0.08	0.06±0.01	0.15±0.11	0.48	0.13	0.80
Total Omega-6	2.12±0.79	0.71±0.47	1.98±0.55	3.51±1.75	0.95	0.17	0.14
C18:2n6c/ LA	0.32±0.17	0.12±0.07	0.09±0.08	0.13±0.11	0.40	0.21	0.10
C18:3n6/ GLA	0.11±0.06	0.13±0.04	0.12±0.05	0.08±0.05	0.89	0.68	0.61
C20:2n6/ Eicosadienoic acid	0.31±0.10	0.31±0.15	0.19±0.11	0.17±0.14	0.92	0.47	0.88
C20:4n6/AA	$0.01±0.01^{a}$	$0.10±0.03^{b}$	$0.11± 0.06^{b}$	$2.29±2.20^{c}$	0.10	0.08	**0.03**
C20:3n6/ DGLA	1.36±0.44	1.31±0.64	1.42±0.26	0.83±0.66	0.54	0.68	0.60
Total Omega 3	0.49±0.27	0.25±0.04	0.10±0.17	0.44±0.26	0.55	0.72	0.61
C18:n-3/ALA	0.15±0.10	0.07±0.05	0.14±0.02	0.18±0.11	0.99	0.74	0.37
C20:3n3/Eicosatrienoic acid	0.04±0.04	0.06±0.03	0.01±0.10	0.17±0.12	0.35	0.63	0.56
C20:5n3/ EPA	$0.01±0.05^{a}$	$0.05±0.01^{b}$	$0.20±0.01^{c}$	$0.02±0.20^{a}$	0.63	0.08	**0.03**
22:6n-3/ DHA	0.29±0.21	0.08±0.03	0.46±0.18	0.06±0.50	0.96	0.49	0.26

Table 4. 5 The fatty acid profile in the uterine after varying ratio LA: ALA followed with different level of fat diet during pregnancy. The group of diet were high ALA and high fat diet (1:1.5 36%), high ALA and low fat diet (1:1.5 18%), high LA and high fat diet (9:1 36%), high LA and low fat diet (9:1 18%). OA : Oleic Acid, LA: Linoleic Acid, GLA: Gamma Linoleic Acid, DGLA: Dihommo gamma linoleic acid, AA : Arachidonic acid, EPA : Eicosapentaenoic acid, ALA : Alpha Linolenic acid, DHA : Decosahexaenoic acid. Values are means±SEM. Statistic analysis was used Two way Anova Test, with different superscript letters signify significant differences at the $P<0.05$.

4.3.2 The effects of dietary omega-3 PUFA consumption on protein expression of Contractile Associated Protein (CAPs).

The different ratio of omega-3 and omega-6 PUFA consumption and level of fat did not affect expression of Oxytocin Receptor (OXTR) CX-43 and PCX-43/ CX-43 (see figure 4.2). However, increasing ALA consumption during pregnancy significantly increased CX-43 and Cav-1 ($P<0.05$) (Figure 4.1). The (1:1.5 36%) group had two times higher expression of Cav-1 compared to (9:1 18%) group. There was no significant interaction between diet and the level of fat for CX-43, Cav-1 and COX-2 expression. Interestingly, the level of fat affected COX-2 expression ($P\leq0.01$). A high level of total fat (36%) increased COX-2 synthesis significantly when compared to the low total fat level (18%). There were no differences in COX-2 expression between the 1:1.5 36% and 9:1 36% dietary groups, but COX-2 expression in 1:1.5 36% group was almost two times higher than the 1:1.5 18% group. This illustrates that the level of fat plays a role in in COX-2 protein expression.

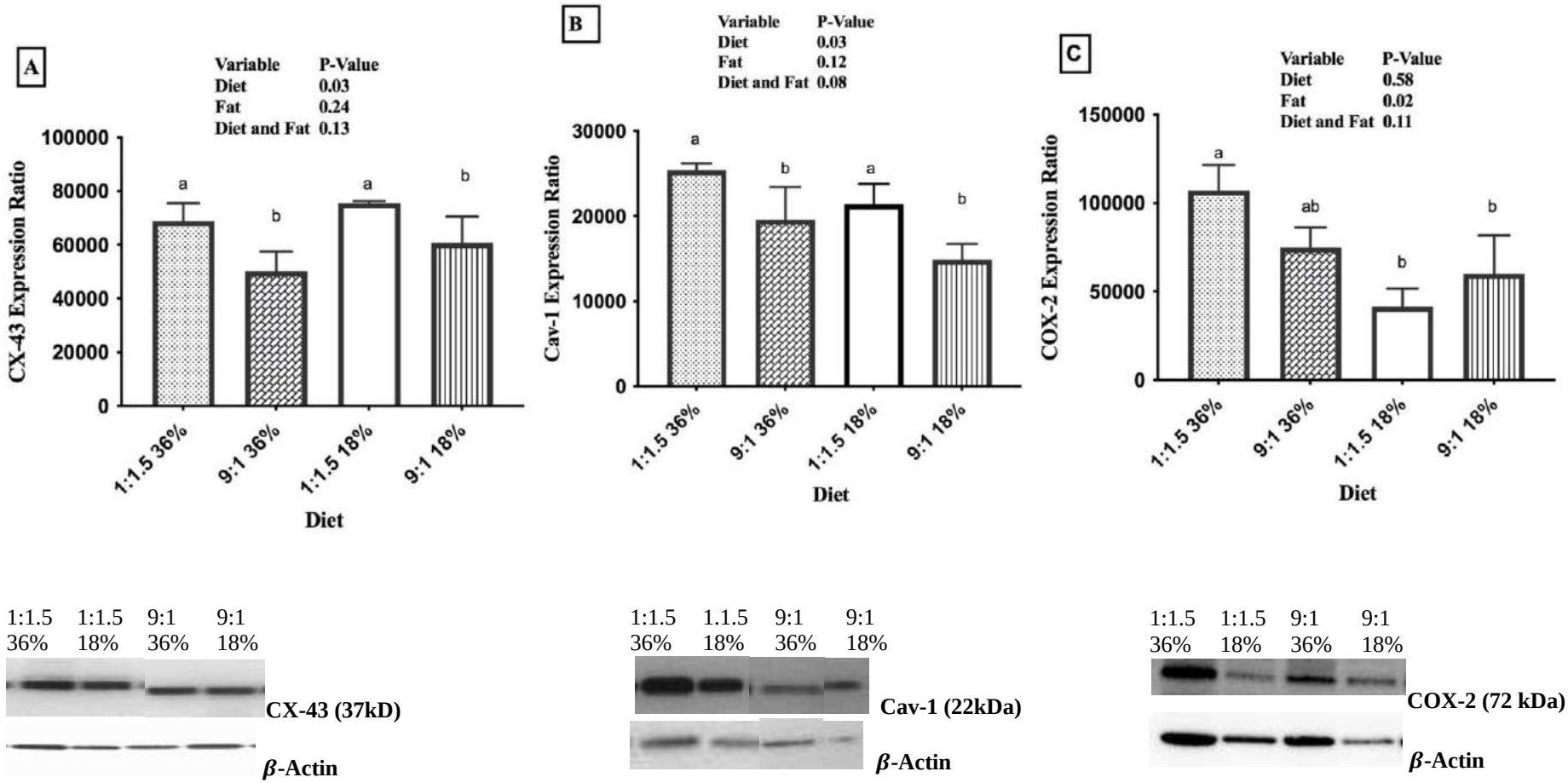

Figure 4. 1. The effect of manipulation of the omega-3 and omega-6 ratio at different fat levels upon contractile associated protein expression in the term labouring rat uterus. (A) Connexin-43 (CX-43), (B) caveolin-1 (Cav-1), (C) Cyclooxygenase (COX-2). Sample size for 1;1.5 36%, 1;1.5 18% and 9:1 18% group were 4, and 9:1 36% group was 5. Values are means±SEM. Statistical analysis was Two way ANOVA test, with LSD test for comparisons. Different superscript letters signify significant differences at the P≤0.05 level.

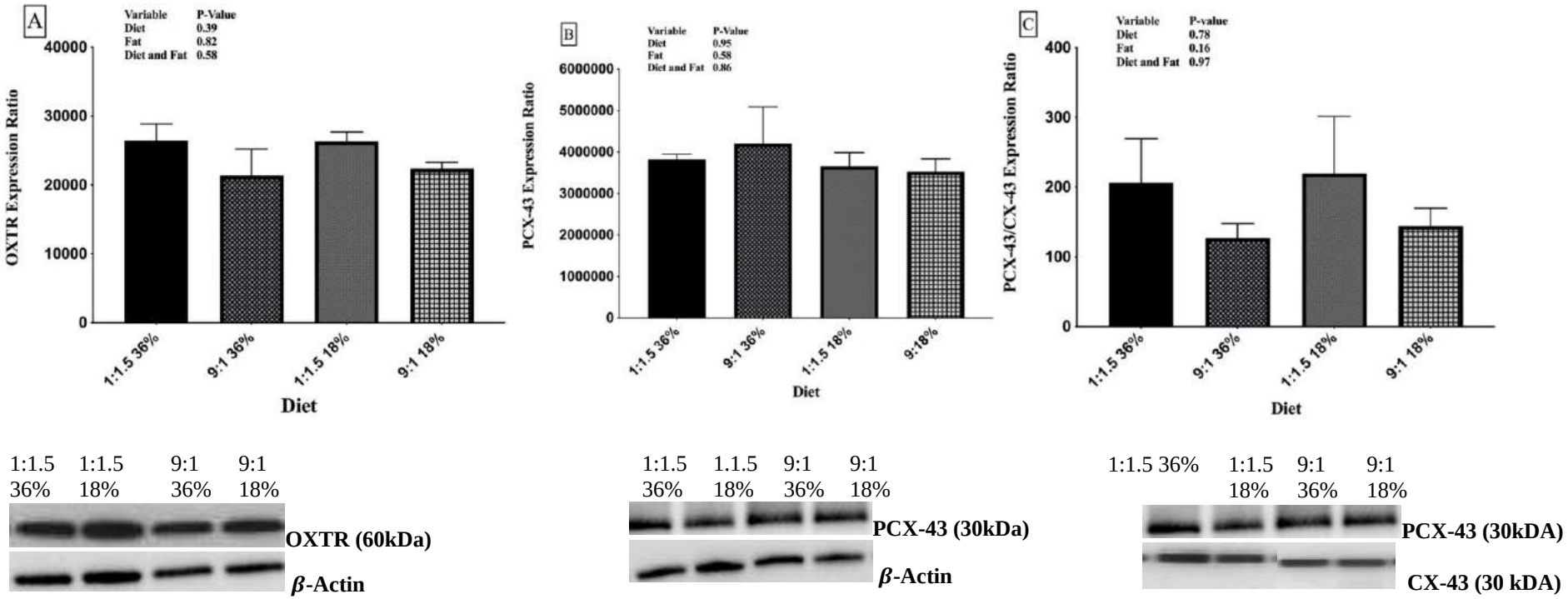

Figure 4. 2. The Effect of manipulation ratio between Linoleic acid (LA) and Alpha-linolenic acid (ALA) followed with different levels of fat upon Contractile Associated Protein (CAPs) in the term labouring uterus. (A) Oxytocin Receptor (OXTR)), (B) Phosphorylation Connexin-43 (PCX-43) (C) Connexin-43 (CX-43) compared with phosphorylation Connexin-43 (PCX-43). Sample size for 1;1.5 36%, 1;1.5 18% and 9:1 18% group were 4, and 9:1 36% group was 5. Values are means ± SEM. Statistic analysis used Two way Anova Test, followed with LSD test. With differences at the P≤0.05.

4.3.3 The effects of dietary omega-3 PUFA consumption on protein expression of Peroxisome Proliferation Activated Receptors alpha, delta and gamma (PPAR-α, PPAR-δ, PPAR-λ)

The uterus from the high LA high fat (9:1 36%) exposed rats had the highest expression of PPAR-α. The high LA, high fat group (9:1 36%) had 30% higher PPAR -α expression compared to the high ALA high fat group diet (1:1.5 36%). Interestingly, PPAR-α expression was not statistically different between high LA, high fat (9:1 36%), and high ALA low fat (1:1.5 18%), suggesting that only the interaction between diet and level of fat significantly affected PPAR-α expression (Figure 4.3).

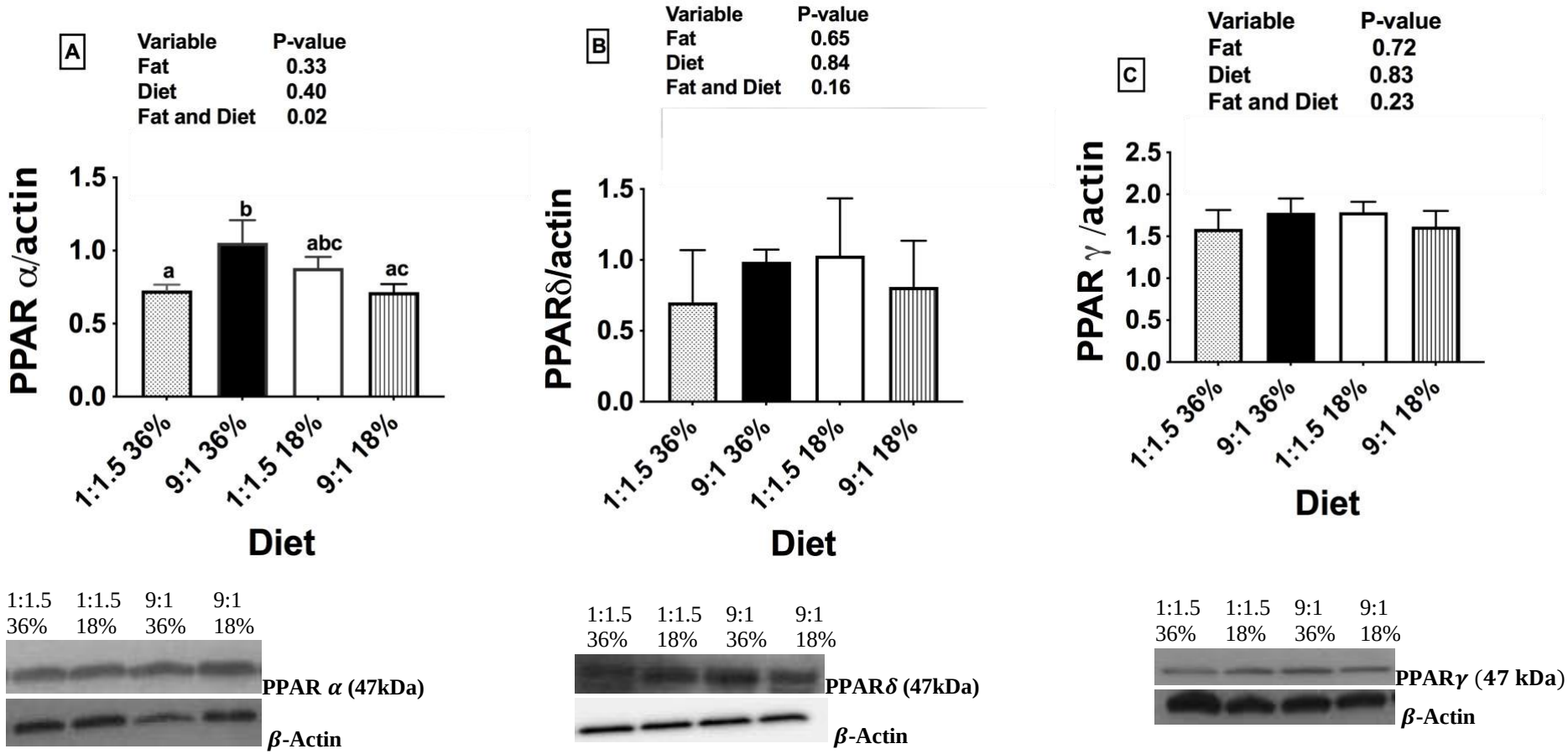

Figure 4. 3 The effect of manipulation ratio between omega-3 and omega-6 followed with different level of fat upon peroxisome proliferator activated receptor (PPARs) in the term labouring uterus. (A) PPARα, (B) PPARδ, (C) PPARγ . Sample size for 1;1.5 36%, 1;1.5 18% and 9:1 18% group were 4, and 9:1 36% group was 5. Values are means±SEM. Statistic analysis used Two way Anova Test, followed with LSD test with difference superscript letters signify significant differences at the P≤0.05

4.4 Discussion

What is evident from current study is that exposure to a high omega-3 diet during pregnancy significantly increased omega-3 PUFA levels through increases in ALA, EPA and DPA and decreased total omega-6 PUFA including, LA and AA proportion in the plasma. However, the uterus only differed in the level of AA and EPA which highlighted that increasing omega-3 PUFA and omega-6 consumption during pregnancy induced very little change of fatty acids in the term uterus. This might suggest that the uterus was more resistant to changes in dietary fatty acid and required for use during labour. This theory was supported by the previous study that mentioned changing from a control to HFHC diet during pregnancy only decreased the total omega-3 PUFA in the labouring rat uterus (Muir et al., 2018).

Interestingly, the interaction between diet and fat significantly affect AA and EPA where in this study mentioned that high fat diet and high LA significantly decreased AA, while high fat and high ALA diet significantly decreased EPA. This suggest that fat might contribute to decreased AA and EPA proportion in the uterus. This result was similar with previous study (chapter 3) that high fat diet during pregnancy significantly decreased omega-3 and omega 6 in the plasma and liver, and only decreased omega-3 in the uterus. The 36% fat diet may have contributed to reduced desaturase, particularly delta-5 desaturase and delta 6-desaturase then lead to decreased EPA and AA proportion in uterus. This theory supported by Wang et al (2006) that stated obesity may negatively impact PUFA desaturation and elongation process. From this study also illustrated that mice fed high saturated fat diet reduce hepatic Fads1 and Fads 2 gene expression as well as Elovl5 expression and activity. The delta- 5 desaturase (D5D) enzymes is encoded by Fads 1 which was involved in

conversion of Eicosastearic acid to EPA and DGLA to AA, while Δ6-desaturase is encoded by Fads2 (Muzsik et al., 2018). The study reported that genetic variations of FADS1 alters D5D index and PUFA status especially ALA, GLA, DGLA, EPA and AA levels in human whole blood (Wolters et al., 2017), while Elovl5 is required to convert GLA to DGLA (Patterson et al., 2012). Another study provide the evidence of decreasing fads1 and fads2 also Elovl5 gene expression in the mice after 12 weeks fed high saturated diet (Picko and Murphy, 2016). The negative impact of high fat diet on the delta-5 desaturase and delta-6 desaturase might occurs in the uterus and potential to decrease ALA proportion in the uterus.

The data from the current study illustrated that the different ratio of omega-3 and omega-6 PUFA fed at 2 different fat levels had no significant effect on protein expression of PCX-43 and OXTR. However, a diet enriched with omega-3 PUFA significantly increased protein expression of CX-43 and Cav-1 and could potentially increase myometrial contractility. A recent study conducted by Radosinska et al., (2015) identified that omega-3 treatment increased mRNA expression of CX-43 and increased abundance of immune-blotting of CX-43 in the rat heart. The same could occur in the uterus, whereby increased omega-3 PUFA increases gap junction integrity potentially improves coordinated myometrial contractions. CX-43 is the major gap junction protein that facilitates electrical and metabolite communication between coupled cells (Solan and Lampe, 2005). Communication between cells within an organ is important to ensure proper function of an organ. The gap junction is important to a number of biological cells, including rapid transmission of electric signals to coordinate contraction of smooth muscle cells, the intercellular propagation of Ca^{2+} waves and synchronization of cell activities (Dlugoslova et al., 2009). Therefore, CX-43 is essential for synchronous contractions in uterus during expulsion of the fetus during

labour. These findings are supported by Doring et al., (2006) who reported that the deletion of the CX-43 coding region in myometrium significantly prolonged the process of birth.

The omega-3 PUFAs, especially DHA and EPA, play a vital role in restoring the expression level and distribution of CX-43 within the cell membrane (Dlugosova et al., 2009; Fischer et al., 2008). Omega-3 PUFA incorporation into cell membranes modify the physical properties of the lipid microdomains and protein and cellular function. Langelier et al., (2010), identified that protein level of CX-43 in the lipid rafts of neural stem cell cultures was increased after DHA supplementation. If the same mechanism occurs in the uterus, it will potentially improve myometrial contractile activity with obesity.

The current study also showed that Cav-1 expression was increased significantly in rats fed the higher omega-3 PUFA diet compared to the high omega-6 diet and has the potential to synchronize contractions during parturition, as it has been reported that Cav-1 indirectly stimulates smooth muscle contractility through inhibition of RhoA and ROK translocation which is necessary for Ca^{2+} sensitization and increases rat and human uterine smooth muscle cell contractile force (Lee et al., 2001b; Taggart, 2001). Furthermore, disruption of the lipid bilayer and caveolae where Cav-1 localize is associated with increase in maxi-K channel and decreases synchronization of uterine contractions in human myometrial smooth muscle cells (Brainard et al., 2005; Brainard et al., 2009). Human umbilical endothelial cells exposed to increased omega-3 PUFA (EPA) concentrations have been shown to cause greater distribution and expression of Cav-1 than cells exposed to Stearic acid (Li et al., 2007) similar to the finding in this study where uterine Cav-1 protein expression was increased after omega-3 treatment.

An interesting finding of the current study was that diet did not affect COX-2 protein expression, but a high fat level did significantly increase COX-2 protein expression. This result suggests that a diet high in ALA diet stimulated an increase in COX-2 protein expression but only if consumed at a high level and that the level of fat might have an important role in regulating protein expression of COX-2. The evidence that fat can increase protein COX-2 in rat myometrial tissue is also support by Elmes and colleagues (2011) who reported significantly higher protein expression of COX-2 at term labour in the rat that fed with high-fat, high-cholesterol diet during pregnancy compared with control diet. The up-regulation of COX-2 is essential for prostaglandin type-2 synthesis, including PGE_2 and $PGF_{2\alpha}$ during parturition (Smith et al., 1991a). Some animal and cell studies provide evidence that increasing $PGF_{2\alpha}$ through COX-2 upregulation at term labour was essential to induce myometrial contractions (Challis et al., 2002a; Gross et al., 2000; Xu et al., 2013). Therefore, increasing protein expression of COX-2 with a high omega-3 36% fat level looked like a promising prospect to synchronize myometrium contraction at term labour in obese pregnant women.

However, in the current study we found no correlation of protein expression of OXTR between any of the dietary groups. A previous study supported these findings as no differences in mRNA expression of oxytocin receptor with maternal intravenous treatment with omega-3 PUFA during late gestation (Ma et al., 2000). The current study therefore suggested a beneficial effect of omega-3 PUFA consumption on the process of parturition through increasing protein expression of CX-43 and Cav-1. At the same time, the level of fat could contribute in increasing the protein expression of COX-2. The omega-3 PUFA consumption has the potential to improve myometrial contractions during labour and prevent dysfunctional labour often associated with obese pregnant women.

Furthermore, the current study provides evidence that manipulation of the ratio of omega-3 and omega-6 PUFAs and the level fat altered uterine protein expression of PPARα. However, PPARγ and PPARδ were not significantly different between all experimental groups. An immunohistochemistry study provide evidence that PPARα located in the nucleus of uterine smooth muscle cells (Dong et al., 2013), while PPAR δ was highly expressed in the implantation site of the rat uterus and in decidual cells and that PPARγ highly expressed in placental tissue at various stages of gestation (Lord et al., 2006). Furthermore, a PCR study revealed that PPARα and PPARδ transcripts are expressed in bovine endometrial stroma cells within uterine tissue (Sheldrick et al., (2007). This suggests that PPARs play different roles in pregnancy, as mentioned by Dong et al., (2013) that reports an important role of PPARα in labour suppression and maintenance of uterine quiescence through anti-inflammatory inhibition in human myometrial tissue, while Barak et al., (1999 and 2002) reports that PPAR γ is essential for differentiation of the placenta and PPAR δ seems to be more important for normal development of the placental-decidual interface.

The exciting finding of this study was that PPARα expression was significantly increased by the high LA 36% fat group (9:1 36%) and was 40% higher than high LA 18% fat group (9:1 18%). This provides evidence that the level of fat might regulate uterine protein expression of PPARα. This finding is supported by a previous study that shows increasing WY14643, marker genes involved in PPARα signalling in the rat liver after treatment with high levels of saturated fat (Patsouris et al., 2006). The same mechanism of increasing PPARα signalling after increased saturated fatty acid exposure may occur in the uterus. Moreover, the high fat diet which was associated with obesity and known to increase free fatty acids (FFA) and β oxidation via the intervention of PPARsα activation (Pawlak et al., 2015; Sikder et

al., 2018). This could be the reason behind the increase in expression of PPARα in the high omega-6 with 36% fat groups.

The increased protein expression of uterine PPARα potentially decreases myometrial contractile activity during parturition through COX-2 suppression (Slattery et al., 2001). In addition, suppression of COX-2 activity also leads to decreased synthesis of prostaglandin type-2 (PGE_2), an enzymes which stimulate myometrium contraction in rabbit uterine cells (Sato et al., 2001).

Another potential mechanism through which PPARα may increase relaxation of the myometrium during labour is through suppression of pro-inflammatory markers such as NfkB and IL-1β (Korbecki et al., 2019). Nf-kB and IL-1β are pro-inflammatory agents that play a critical role in stimulating arachidonic acid release and increase expression of protein and mRNA COX-2 in human study (Korbecki et al., 2019; Rauk and Chiao, 2000). This theory is supported by Dong et al. (2013) who provide evidence of a negative correlation between protein and mRNA PPARα and IL-1β in the human myometrium. The same study also highlighted that upregulation level of PPARα play an essential role in maintaining pregnancy by inhibiting myometrial contractions via suppression of IL-1β. PPARα activation inhibits IL-1β gene transcription via interference of NfkB and activator protein-1 (AP-1) pathways in the human aortic smooth muscle cells (Steals et al., 1998). The same mechanism may potentially occur in the uterine smooth muscle cells, particularly as a PPARα knockout study suggests that PPARα maintain uterine quiescence by stimulating mRNA TH2 cytokine which inhibits COX-2 in pregnant mice (Yessoufou et al., 2006).

It is clear from the results that a diet high in omega-3 PUFA increased the protein expression of the CX-43 and Cav-1, also interaction existed between the high ALA and high fat diet to increase protein expression of COX-2. Prasad et al. (2010)

identified that the membrane fluidity of mice thymocytes was higher following administration of omega-3 compared to omega-6 FA's. Increased membrane fluidity enhances receptor signalling and coupling efficiency particularly G-protein coupled receptors (including FFAR-1 and FFAR-2) that is in turn associated with higher levels of proteins that facilitate contraction such as MAPK and connexin (Poebla et al., 2017). Furthermore, review studies found that the binding of caveolins with G-protein coupled receptor and RhoA could induce Ca^{2+} release from SE and potentially increase contraction (Okamoto et al., 1998).

High fat diets often associated with obesity are characterised by broad inflammatory responses in both animals and humans. Immunohistochemical analysis shows that inflammation induces COX-2 expression in the endometrial tissue of horses (Segabinazzi et al., 2017). The same mechanism of increased protein expression of COX-2 after either a high omega-3 high fat (1:1.5, 36%) or omega-6 high fat (9:1, 36%) potentially occurs in the uterus of pregnant rats as well as increased strength of contraction since COX-2 is an important enzyme for prostaglandins production. Slater et al. (1999) demonstrated evidence of increased COX-2 mRNA and protein levels before increased prostaglandin synthesis in human myometrium at term labour. The effect of varying the ratios of omega-3 and omega-6 with different levels of fat can be seen in Figure 4.4.

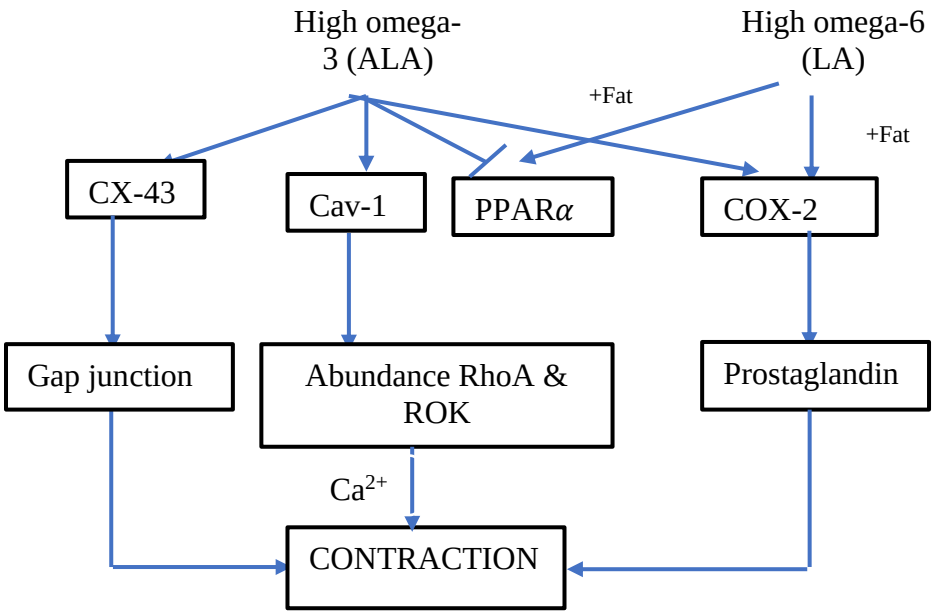

Figure 4. 4 The effect of Omega-3 ALA (alpha linolenic acid) and Omega-6 LA (Linoleic acid) also High omega-3 high fat (1.1.5 36%) and high omega-6 high fat (9:1 36%) on PPARα: Peroxisome proliferation activated receptor α, Contractile associated protein (CAPs) such as CX-43 : connexin-43, Cav-1:Caveolin-1, Cyclooxygenase-2 : COX-2. RhoA : Ras homolog A, ROK : Rho associated protein kinase.

4.5 Strength and Limitation of study

It was clear that exposure to high levels of LA and ALA at different fat levels alters fatty acid composition in the plasma and uterus. From this study also proved that high fat diet contribute to decrease EPA and AA in the uterus that suggest maternal obesity potential to supress elongase and desaturase activity since Wang et al., (2006) reported decrease protein expression and activity delta-5 and delta-6 Desaturase and Elovl 5 and Elovl 2 in hepatic diabetes obese rat. Furthermore, increased omega-3 in the diet was associated with increased protein expression of CX-43 and Cav-1, and that interaction between high omega-3 and high fat contributed to increased COX-2 but decreased PPARα. This study illustrates that omega-3 PUFA consumption could be a potential treatment in reducing prolonged and dysfunctional labour with maternal obesity,

Although the data suggests omega-3 PUFA could improve labour outcomes with maternal obesity there are a number of limitations that have to be noted. The first

is to consider that the absolute fatty acid concentrations within the plasma and uterus was not quantified just as a percentage of the total fatty acids. Moreover, The proportion of uterus monounsaturated fatty acid, omega-9, omega-3 and omega-6 detected very little which caused total fatty acid was not 100%. There was some reason behind those fatty acid only detect very small such as any contamination in the uterus tissue from previous study or any reagents were impure this could introduce other compound then detected by the FID. Another reason is monounsaturated and unsaturated fatty acid might lose during oxidation when sample allow to dry out during analysis, also possible the layers were mixed slightly after centrifugation during the extraction on the top layer in the derivatization step. However, although the PUFAs were detected in small percentage but increased the percentage of AA in high LA low fat (9:1 18%) and increased the proportion of EPA in rat fed high ALA and low fat diet (1:1.5 18%) were the evident that interaction between diet and fat contribute to affect proportion of EPA and AA in the uterus. Furthermore, this study only investigate the proportion of fatty acid in the term rat. It would be ideal for future samples to be collected before pregnancy, during gestation and during parturition, with analysis focusing on fatty acid composition of the phospholipid fraction in the uterus. This will provide a better perspective of the effect of different omega-3 and omega-6 diet followed by different levels of fat on the circulatory, hepatic and uterine fatty acid profile.

This study does provide evidence that a high omega-3 diet increases CAPs expression in the rat uterus and has the potential to improve myometrial contractile activity in obese pregnancy. However, no evidence was available to show the effect of a diet high in omega-3 PUFA on CAPs activity in the obese myometrium pregnant rat. Therefore, future studies would be useful to investigate the activity of CAPs, especially Cx-43 hemichannel activity through cell culture techniques. The omega-3 PUFA can

be added to myometrial cells of obese pregnant rats to determine the impact of omega-3 PUFAs on myometrial contractions with maternal obesity. For that purpose, Immunofluorescence can be used to analyse the abundance of CX-43 and Cav-1. Furthermore, CX-43 hemichannel opening is associate with the increasing of intracellular Ca^{2+} in human pregnant myometrial and endothelial cell (Saez et al., 2018; Nadeem et al., 2017). Further evidence reported by Challis et al., (2000) and Hendrix et al., (1995) explain that oestrogen is essential for transport CX-43 to the membrane dan increase assembly of CX-43 into gap junction in plasma that required for effective synchronization of myometrium contraction. Investigation the impact of omega-3 in CX-43 activity through enhance of oestrogen and intracellular Ca^{2+} in myometrial obese pregnant rat cell might provide clearly pathways behind dysfunctional labour in maternal obesity.

5. The effect of oleic acid on *ex-vivo* myometrial contractile activity

5.1 Introduction

Data from the chapter three clearly illustrated that the plasma and hepatic levels of Oleic acid (OA) was significantly affected by the HFHC diet. OA was shown to be significantly higher in the plasma and liver of rats fed the HFHC diet when compared to chow-fed controls, but also in those rats switched from a control diet to a HFHC diet at conception. The study conducted by Fujiwara et al. (2015) supports our data as liver OA levels are increased in obese female cats. Furthermore, it has been reported that mice fed a high fat diet particularly saturated fat until day 7 shows an increasing ratio of stearic acid: palmitic acid (C18:C16) and higher levels of OA compared to day 1 (da Silva-Santi et al., 2016). Another study conducted by Wentzel et al. (2019) also support our findings that intake of a high fat diet during the last trimester of pregnancy significantly increases plasma and liver OA levels in pregnant rats. High fat and high cholesterol diets have also been shown to be strongly associated with obesity and spontaneous preterm delivery when babies are born less than 32 weeks of gestational age compared to women of normal BMI, with an OR 2.99 (Cnattingius et al., 2013).

The previous study that shows increased proportion of plasma and liver OA after high fat feeding during pregnancy in the rat illustrates a potential role for OA altering the process of parturition through PKC activation. A report from Guerra et al., (1991) identified that OA induced PKC translocation from cytosol to the membranes in isolated hepatocytes cells. Thrombin caused the release of OA from phospholipids and could induce sufficient OA available in intracellular and potential to increase concentration of DAG and intracellular Ca^{2+} where this condition induce translocation PKC from cytosol to membrane. From the same study also illustrates that OA prefentially translocated the

β isoenzymes PKC to the membrane, and this isoenzyme is independent of calcium requirement, while α isoenzymes of PKC requires Ca^{2+} to be catalytically active.

Another study conducted by Shinomura et al., (1991) provides further evidence that OA can activate cytosolic PKCα, β, and γ purified from the rat brain in the presence of 1 μM Ca^{2+}, with phosphatidylserine or both phosphatidylserine and Ca^{2+}. Published work by Khan et al., (1992) revealed that 30% of OA in the free form can increase phosphorylation and activation of PKC in the cytosolic compartment of intact human platelets. The same study also identified that OA partially activates calcium-dependent PKC such as PKCα, β and fully activates calcium-independent PKC, such as PKCδ in human platelets. From the same study study also mentioned that initiation of Phospholipase C potential to generate free OA which involve in the activation of PKC family in the rat brain. The activation of Phospholipase C causing the hydrolysis of membrane phospholipid and the subsequent generation of DAG and free fatty acid respectively including free OA which undergo a rapid distribution between membrane and cytosol. The soluble free OA is able to activate soluble protein kinase C (stippled), while inactive soluble PKC is a target for activation by free OA and is presumed to be in equilibrium with membrane-bound protein kinase C which is then catalytically activated by membrane DAG with the presence of calcium. (Khan et al., 1992; Moran et al., 2014 ; Kishimoto at al., 1980; Takenawa et al., 1982). The mechanism through which OA exposure promotes increased PKC could potentially be occurring in the rat uterus and affect myometrium contraction at term labour.

Interestingly, OA has been shown to induce gap junction disassembly through PKCε activation in cultured rat cardiomyocytes (Huang et al., 2004). The same mechanism of OA inducing gap junction disassembly could occur in the uterus and be the cause of poor myometrial contractile activity in obese pregnant women during labour. This theory

is supported by data provided by Muir et al., (2016) showing gap junction disassembly being associated with unsynchronized contractions in maternally obese rats. As a result, this might explain how high OA levels could potentially inhibit myometrial contractile activity via alteration of PKC activity. In contrast, it has been identified that increasing PKCβ activity increase K^+ stimulated muscle force and Ca^{2+} sensitivity of the contractile element within human myometrium (Ozaki et al., 2003). Another study supports this theory that mentioned activation of protein kinase C by phorbol myristate acetate (PMA) increases Ca^{2+} and improves contractions in pregnant rat myometrial cells (Shimamura et al., 1994). These findings suggest that PKC can stimulate or inhibit myometrial contractions depending on the PKC isoform .

Previous research has investigated the effects of PKC on myometrial contractile activity by using a PKC activator or inhibitor to determine their effects on the contractile properties. It was identified that phorbol esters directly activate both conventional and novel PKC isozymes, while Go983 preferentially inhibits PKC α, PKC β and PKC δ (Massenavette et al., 2017). A further study identified that exposure to phorbol esters increases muscle tension of human pregnant myometrial strips and that suggests potential regulation of PKC increases myometrium contraction. In addition, western blot analysis also revealed greater protein expression of PKC β and CPI-17 which are substrate of PKC that might stimulate myometrial contractions during parturition (Ozaki et al., 2003).

There is a clear need to evaluate the effect of OA on parturition and identification of the potential mechanism through which OA may alter myometrial contractile activity with maternal obesity. Investigation of the direct effect of exposure of OA on *ex-vivo* myometrial contractions in the term pregnant rat will help us to discover whether OA could be having adverse physiological effects on myometrial contractile activity and possibly the causal effect of prolonged and dysfunctional labour associated with maternal

obesity. With evidence to suggest that OA increases in obese pregnant women it could be a potential mechanism behind prolonged labour via OA activation of Protein Kinase C (PKC) (Hag et al., 2019; Khan et al., 1991; Muir et al., 2016). As a result, the following research aims to focus on the effects of OA on *ex vivo* myometrial contractions, in addition, whether the effects occur through PKC. This will be achieved via manipulation of the PKC signalling pathway through myometrial exposure to the PKC activator (PMA) and inhibitor (Go6983) respectively.

To achieve this, organ bath techniques will be used to assess whether there is any correlation between the level of OA uterine strips are exposed to and their contractile activity. Uterine strips from term pregnant or laboring rats mounted in organ baths can be used as tools to measure functional responses to pharmacological reagents. This *ex vivo* technique allows for investigation of the contraction performance of the myometrium and measure direct effect of different agents on the parameters of contraction. Dose response curves to OA along with therapeutic agents to inhibit or stimulate PKC were carried out to investigate whether OA exerts its effects on myometrial contractile activity via the PKC signaling pathway.

5.1.1 Objectives and Hypothesis

The objective of this study was to investigate the effect of OA exposure on myometrial contractile activity and to investigate whether PKC has an important role in altering contraction. The hypothesis of the current study was that increasing OA accumulation would decrease myometrial contractile activity, and GO6983 suppresses the effect of OA.

5.2 Materials and Methods

5.2.1 Materials

This experiment used OA, PMA and Go9883 all purchased from sigma ALDRICH. OA was a water soluble form that contained Methyl beta Cyclodextrin, while PMA and G6983 were the pure chemical. All animal experimental work was approved and carried out the Biological Sciences Unit at the University of Nottingham.

5.2 .2 Animal Study

This study used 8 Virgin female Wistar Rats within the animal facilities of the University of Nottingham. All rats were weighed then pair-housed under normal conditions (12 hours light: dark photoperiod, 21°± 5° room temperature, 55% ± 5% relative humidity, food and water access ad libitum. Rats were fed a control chow fed diet that exactly same as in Muir (2016). After acclimatisation in the animal unit, female rats were mated a week with a stud male and pregnancy confirmed upon discovery of a seminal plug which was recorded as gestational day 0. Each pregnant rat was weighed daily to make sure they were putting on weight and also used as an indication that the pregnancy was successful. At 20 days of gestation, the rats were euthanized by rising concentration of CO_2 and cervical dislocation and the foetuses by overdose of pentobarbitone. The uterine horn was then collected and immediately put into ice cold KREBs buffer before carrying out contractile activity measurements in the organ bath.

5.2.3 *Ex-Vivo* contractility study

This experiment was started with modified 1 L fresh KREBs buffer dissolving 119mM NaCl, 4.7 mM KCL, 2.4mM $MgSO_4$, 25mM $NaHCO_3$ and 1.18mM KH_2PO4, 5.5 mM Glucose, 1.6 mM $CaCl_2$ within 1 L dH_2O. From each animal four 10X5mm uterine strips following the circular layer of myometrium were dissected from the

middle of the uterine horn (between the ovary and the cervix) and mounted within a 25 ml organ bath (AD Instrument, Oxford UK) filled with fresh KREBSs buffer and gassed with 95%% O2/5% CO2. Each strip was set to resting tension of 20 mN and contractile activity of each strip was recorded using isometric force transducer connected to bridge amplifier, which in turn was connected to data acquisition system (Powerlab.8SP,AD Instruments, Oxford, UK) which was recorded and analysed by Lab Chart Software (version 6:Powerlab ASD Instruments). Strips of myometrium were then left to equilibrate for 30 to 40 minutes before a 15 minute baseline of spontaneous uterine contractility was recorded prior to accumulative dosing of OA or PKC inhibitor (G06983) or accumulation of OA with PKC activator (PMA) and PKC inhibitor (Go6983).

5.2.4 Effects of OA and PKC activator and PKC inhibitor on uterine contractility

All uterine tissue was left to equilibrate until spontaneous contractions were stabilised for approximately 30 minutes. Once contractions were deemed stable a baseline recording for 15 minutes was completed before increasing concentrations of OA was added into the chamber (0 µM -110 µM). During baseline and dose recordings the frequency of contractions, time to complete a contraction, the average amplitude of the contractions, time to peak contractile activity, peak amplitude, integral activity and maximum slope were measured during each 15 minute exposure to OA dose.

This study investigated the effect of PKC activator and PKC inhibitor on uterine contractile activity. The treatment of OA, PKC activator and PKC inhibitor was done in parallel without washing the uterine tissue with KREBs solution in between treatment processes. In the beginning of this study, the uterine tissue was exposed to an increasing dose of OA dose : 0 μM, 20μM ,30 μM , 40μM, 50μM, 60μM, 70 μM,

80μM, 90 μM, 100 μM,110μM. The 110 μM of OA was considered as the baseline in investigating the effect of PKC activator towards uterine contraction. PMA treatment was consequently added in gradually increasing concentrations of 0.5 μM , 1 μM, 1.5 μM, 2 μM . Subsequently, 110 μM OA and 2 μM PMA activator was used as the baseline in investigating the effect of PKC inhibitor towards uterine contraction. PKC inhibitor (Go6983) was gradually added in increasing concentrations from 0.5uM, 1 μM, 1.5 μM, 2 μM, 2.5 μM, 3 μM, 3.5 μM, 4 μM, 4.5, 5 μM into the organ bath. Uterine contraction data was measured every 15 minutes after the addition of both PKC activator and PKC inhibitor.

To further examine the individual effect of PKC inhibitor on uterine contraction, the author conducted a separate investigation using a smaller number of uterine tissue (2 tissues) in which PKC inhibitor was added, using 0 μM PKC as the baseline. All uterine tissue was left to equilibrate until spontaneous contractions stabilised for approximately 30 minutes, then PKC inhibitor was added gradually from 0 µM until 5 µM and uterine contractions were measured every 15 minutes.

5.2.6 Data analysis

Myometrial contractile activity was extracted from Lab Chart reader software version 8 (AD instruments, New Zaeland). All data were analysed with IBM Statistic 25 software and expressed as the mean value±SEM, with statistical significance, determined at the $P<0.05$ level. The ordinary fit least square analysis was used to determine the correlation between OA dose with myometrial contraction parameters and the correlation between GO6983 exposure with myometrial contraction. Dose response curves were used to analyse the concentration of OA or Go6983 that inhibit 50% of myometrial contractile force (IC50) or stimulate 50% of the contractile (EC50)

force. The dose response curve including IC 50, EC 50 and maximum slope were analysed by dose response-log (agonist/inhibitor)- response-(4 parameters) variable slope using GraphPad PRISM 8 (version 8; Graphpad Inc, San Diego, CA, USA). Meanwhile, The effect of PMA exposure with accumulation with OA and the effect of OA accumulation in cooperation with PMA and Go6983 on myometrial contractile activity were analysed by repeat measured ANOVA.

5.3 Result

5.3.1 Effect of OA treatment on *Ex-Vivo* Myometrial Contractile Activity

Exposing the term pregnant myometrium to increasing concentrations of OA (see **Figure 5.1** and **Figure 5.2**) identified that the peak amplitude of uterine contractions was increased, increasing from 3.5 g of force at baseline (no OA) to 4.2 g after exposure to 110 µM OA exposure. The uterine strip treated with 110 µM OA also increased the length of individual contractions compared to baseline contractions (0 µM OA). However, an increasing level of OA decreased the frequency of contractions. The statistical analysis of the direct effect of exposure to increasing doses of OA on parameters of myometrial contractile activity can be seen in **Table 5.1.**

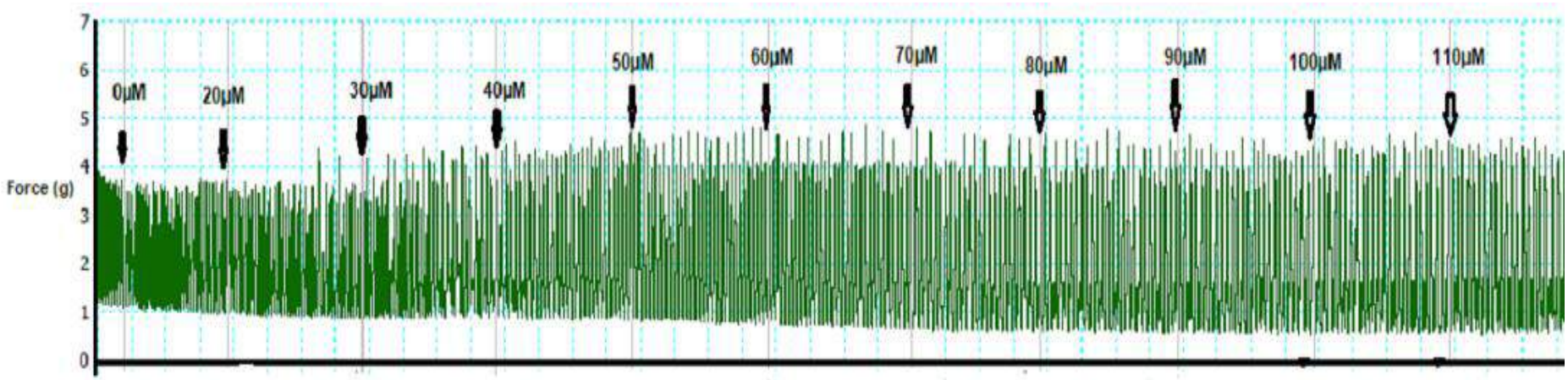

Figure 5. 1 Effect of exposing uterine strips to increasing concnetrations of OA (0 μM -100 μM) on ex vivo uterine contractile activity. Contractile activity was recorded over a period of 15 minutes following each dose.

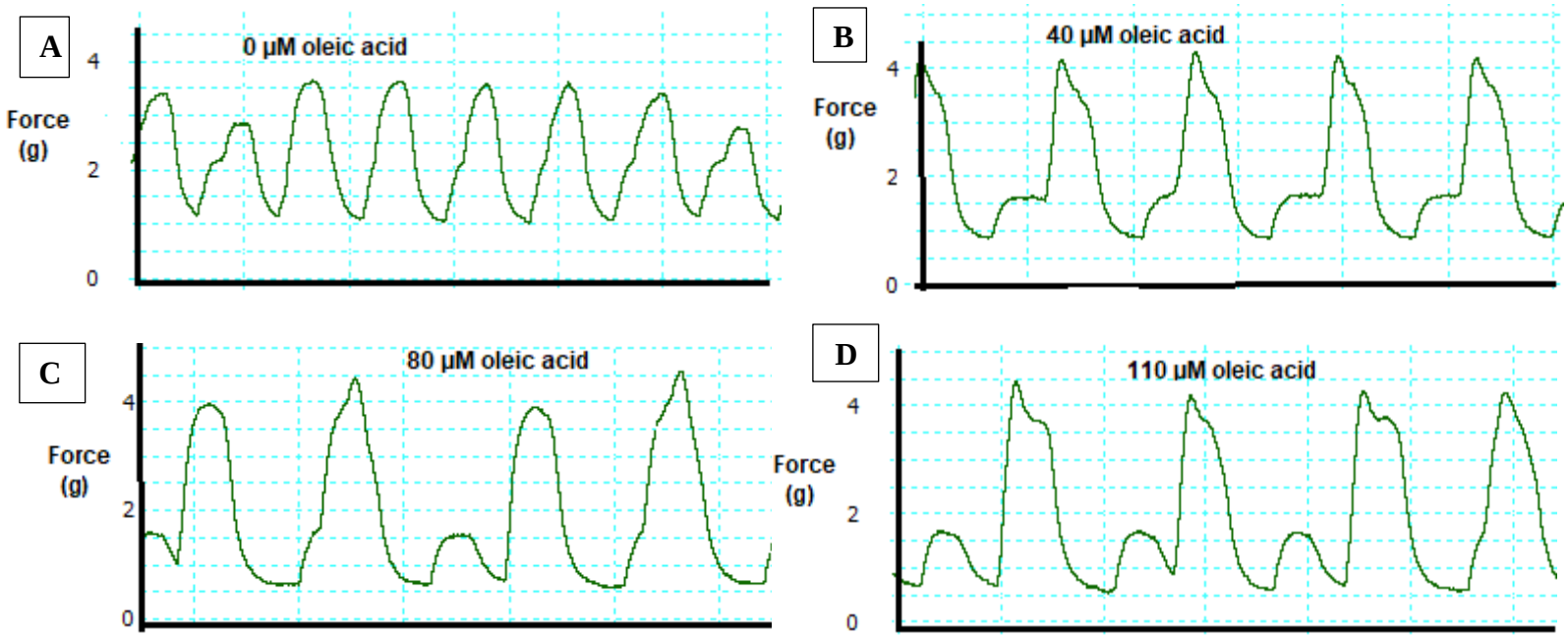

Figure 5. 2 The comparison of OA treatment with concentration (A) 0 uM, (B) 40 uM, (C) 80 uM , (D) 110 uM in myometrial contractile activity ex-vivo. The myometrial contraction activity was recorded in 15 minutes after each dose treatment.

5.3.2 Effect OA treatment in uterine contractile activity

Direct exposure to increasing doses of OA significantly increased the peak amplitude, mean amplitude, time to peak and mean contraction area with P value<0.01 (**Table 5.1**). The OA dose of 110 µM had the longest time to peak contraction, and mean area under the curve also integral activity compared to baseline. The mean amplitude significantly increased almost 100% after 110 µM of OA compared with baseline. Meanwhile, there was no significant difference in maximum slope between baseline and OA treatment (P>0.05). Moreover, increasing OA accumulation increased peak of amplitude, mean of amplitude, integral activity, time to peak, and mean area under contraction in a dose dependent manner. Increasing OA accumulation significantly increased peak of amplitude of contraction with log EC 50 = 1.65 µM (**Figure 5.3**) also increased mean of amplitude and integral activity with log EC50 = 1.67 µM and 1.65 µM (**Figure 5.4**). The maximum integral activity that reached after increased OA accumulation was 763.4 g.s (**Figure 5.5**).

However, instead of increased amplitude, increasing OA concentration significantly inhibited frequency of contractions (P<0.01) with IC 50 = 54.03 µM and maximum frequency 26.03 (**Figure 5.3**). The contractile frequency after 110 µM OA was significantly decreased by 42% compared to baseline (0 µM OA) with P- value < 0.01.

OA dose (μM)	**Frequency per 15 minutes Mean±SEM (P <0.01)**	**Length of Contraction (ms) Mean±SEM (P< 0.05)**	**Mean of Amplitude (g) Mean±SEM (P< 0.01)**	**Time to peak(ms) Mean±SEM (P<0.01)**	**peak of Amplitude (g) Mean±SEM (P,0.01)**	**Mean Area Under Contraction Mean±SEM (g.s) (P<0.01)**	**integral activity (g.s) Mean±SEM (P<0.05)**	**Maximum slope (g/s) Mean±SEM (P>0.05)**
0	26.37±2.83	15870±1720.43	1.65±0.17	4412.13±309.38	3.47±0.27	16.61±3.64	518.81±121.34	3.27±0.03
20	25.63±2.95	15697.50±1581.08	1.78±0.14	4675.38±400.82	3.38±0.26	18.33±4.36	490.57±128.36	3.28±0.04
30	26.88±3.31	17356.25±1950.53	1.88±0.21	4790.86±280.81	3.41±0.19	22.81±5.92	529.30±107.50	3.28±0.04
40	23.38±2.72	17793.75±2213.93	2.44±0.23	4787±199.28	3.77±0.22	24.82±6.32	544.85±108.40	3.31±0.03
50	22.36±2.31	18223.75±2644.90	2.67±0.21	5128.75±607.26	3.8±0.22	28.78±8.21	557.74±98.06	3.34±0.05
60	20.25±2.86	18722.5±2660.51	2.81±0.28	5136±395.60	4.08±0.28	29.57±7.46	576.36±78.35	3.30±0.05
70	19.50±3.04	19928.75±2819.54	3.00±0.39	5340.63±514.50	4.09±0.38	31.50±8.67	622.97±157.10	3.26±0.07
80	18±2.73	20880±3057.09	3.28±0.42	5391.25±363.48	4.12±0.44	34.15±7.16	648.22±109.19	3.11±0.14
90	17.63±2.74	21308.75±3395.43	3.34±042	5558.63±543.18	4.11±0.44	37.10±8.22	682.53±85.31	3.04±0.12
100	17.5±2.71	21322.5±2665.75	3.30±0.31	5564.13±251.17	4.17±0.43	37.22±9.19	697.59±95.72	3.31±0.04
110	15.13±2.62	21456.25±3937.34	3.29±0.42	5551.5±672.12	4.17±0.39	37.40±9.38	704.84±105.47	3.26±0.04

Table 5. 1 Dose response of term uterine horn strips to OA (0 μM to 110 μM) . The ordinary Fit Least Square statistic was used to analyse the correlation between increasing OA dose with responding myometrial contractile activity with significance taken at the P<0.05 level. All parameters of contractile activity was measure after 15 minutes exposure to each dose.. The number of uterine strips was n=8 and values presented as means ±SEM.

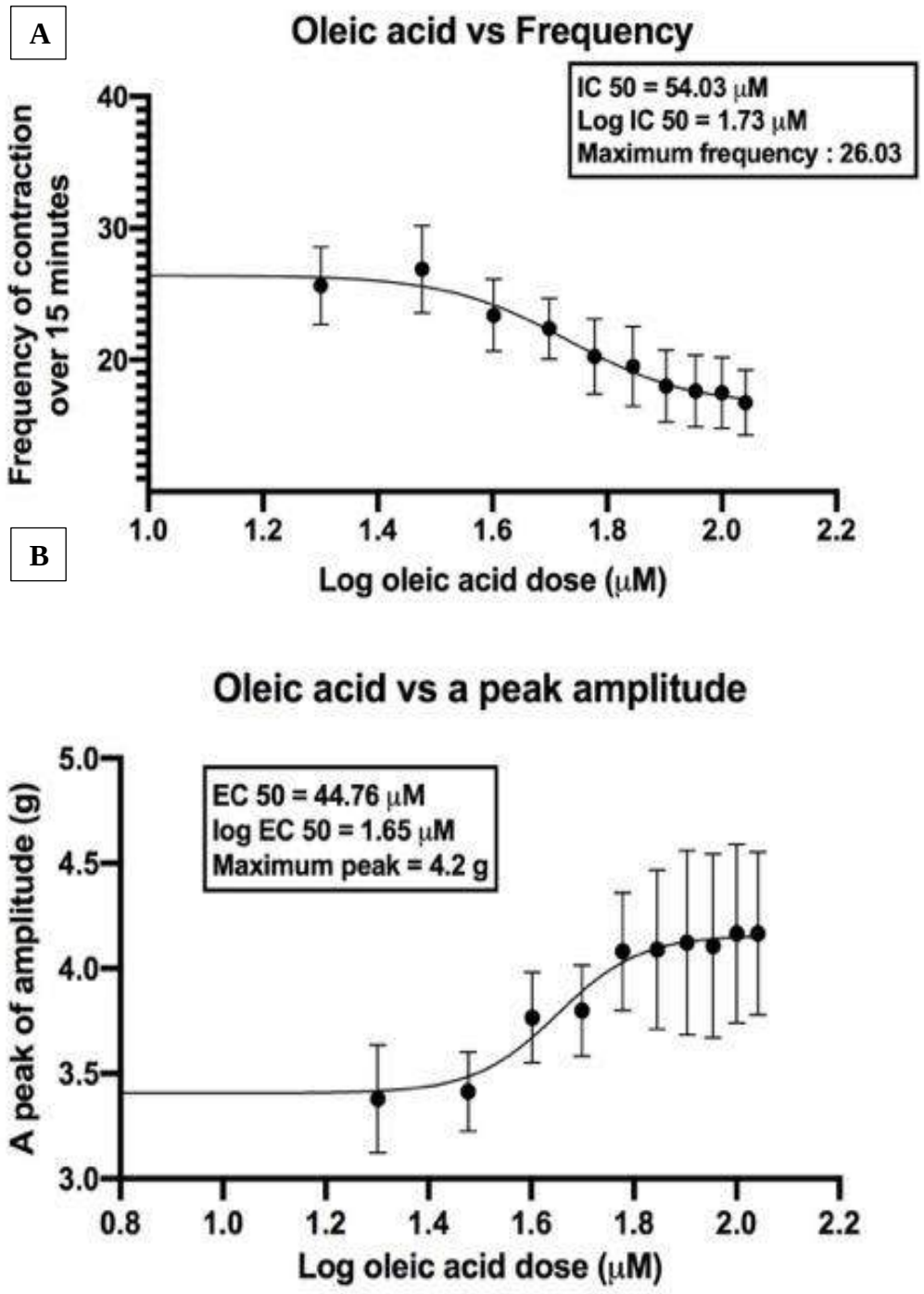

Figure 5. 3 Dose response analysis of term pregnant myoemtrial strips to increasing doses of OA (0-110 μM) . (A) Frequency of contractions (15mins) and (B) Peak amplitude. n= 8 uterine strips . Values are means ±SEM.

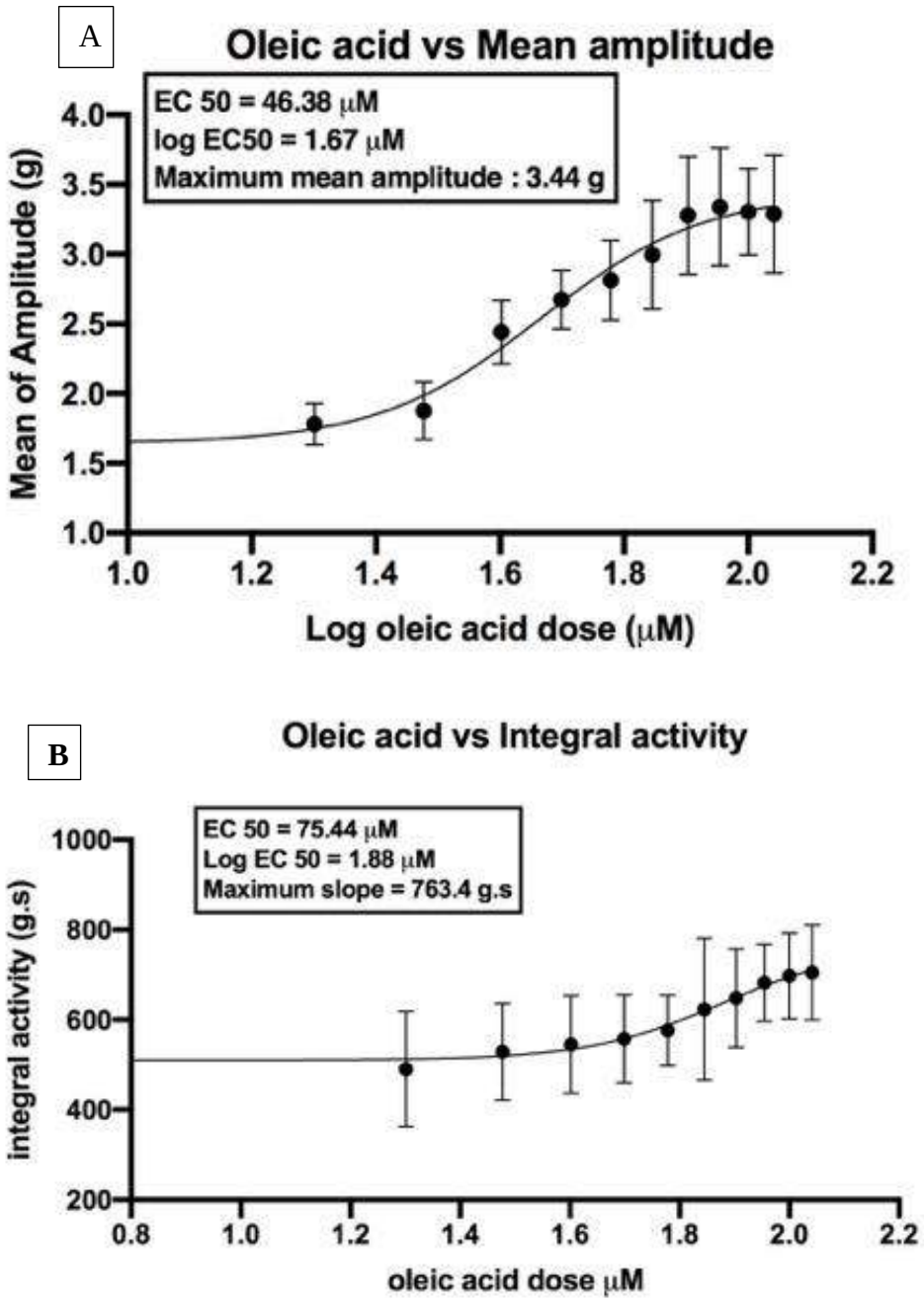

Figure 5. 4 Dose response analysis of term pregnant myoemtrial strips to increasing doses of OA (0-110 μM) . (A) Mean amplitude of contraction and (B) Peak amplitude. n= 8 uterine strips . Values are means ±SEM.

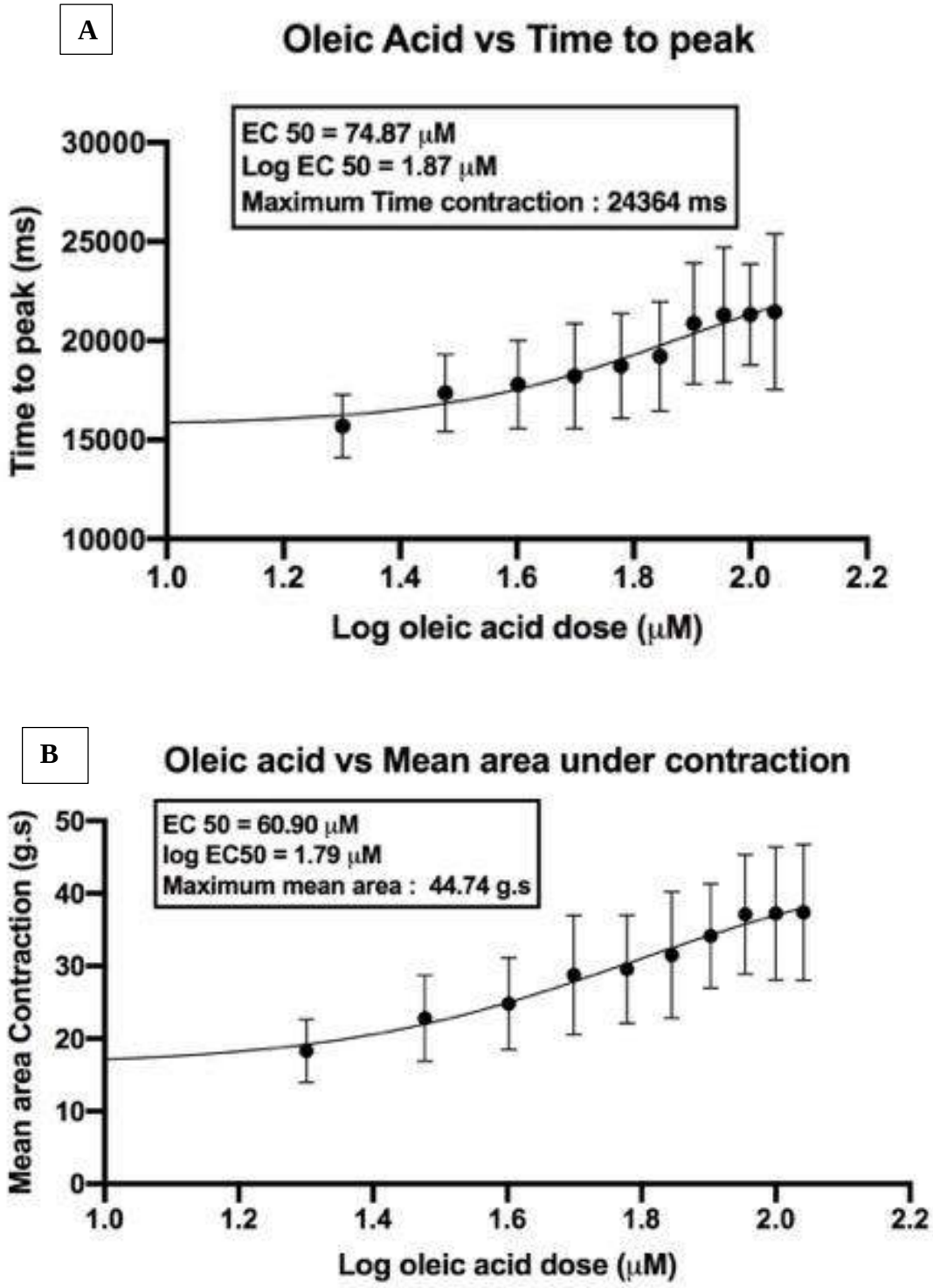

Figure 5. 5 Dose response analysis of term pregnant myometrial strips to increasing doses of OA (0-110 μM) . (A) Time to peak and (B) Mean area under contraction. n= 8 uterine strips . Values are means ±SEM.

5.3.2 Effect of PKC activator (PMA) and PKC inhibitor (Go6983) treatment on *ex-vivo* Myometrial Contractile Activity

Investigation of the effect of PKC inhibitor (Go6983) on myometrial contractile activity identified that exposing uterine strips to increasing dose of the PKC inhibitor Go6983 decreased both the frequency and peak amplitude of contractions ($P<0.01$) **(Figure 5.6).** Moreover, **Figure 5.7** illustrated that increased exposure of Go6983 as PKC inhibitor down regulated frequency of contraction and peak of amplitude from 5 (g) to 3 (g). Furthermore, accumulated of oleic acid exposure with PMA was slightly decreased peak amplitude of contraction, while GO6983 in accumulated with PMA and oleic acid decreased peak of amplitude **(Figure 5.8)**. Data from the current study also identified that uterine contractions after exposure to the highest level of PKC activator (PMA) became synchronized similar to the contractions seen at baseline. However, even though PMA did not significantly affect the myometrial contractile activity of pregnant rat uterine tissue, the peak contractile response decreased slightly with 110 µM PMA treatment. Interestingly, 110µM OA in combination with PMA and Go6983 led to unsynchronized contractions when compared to OA treatment alone and in combination with PMA (**Figure 5.9)**. From **Figure 5.8 and 5.9** it can also can be seen that the peak amplitude of contraction decreased almost 26% after added 5.5 µM Go6983 in combination with 110 µM OA and PMA in comparison to peak amplitude of uterine strips after exposure with 110 µM OA.

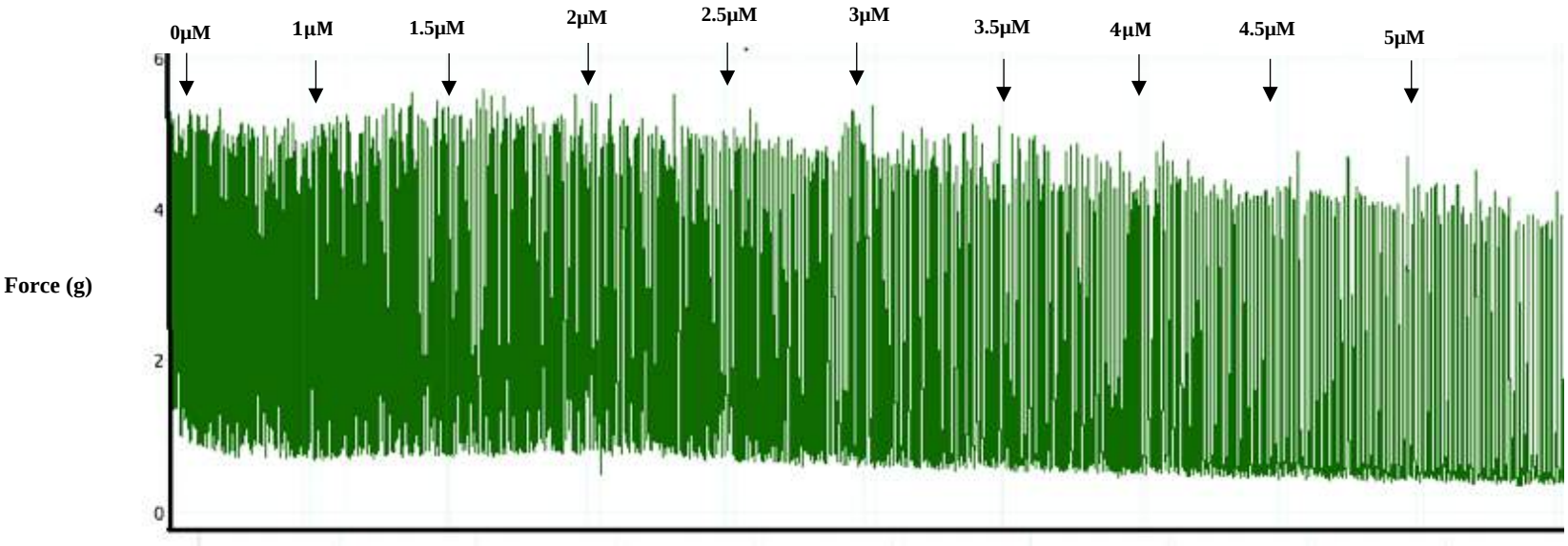

Figure 5. 6 Effect of exposing uterine strips to increasing concnetrations of Go6983 (0 $\mu M - 5$) on ex vivo uterine contractile activity. Contractile activity was recorded over a period of 15 minutes following each dose.

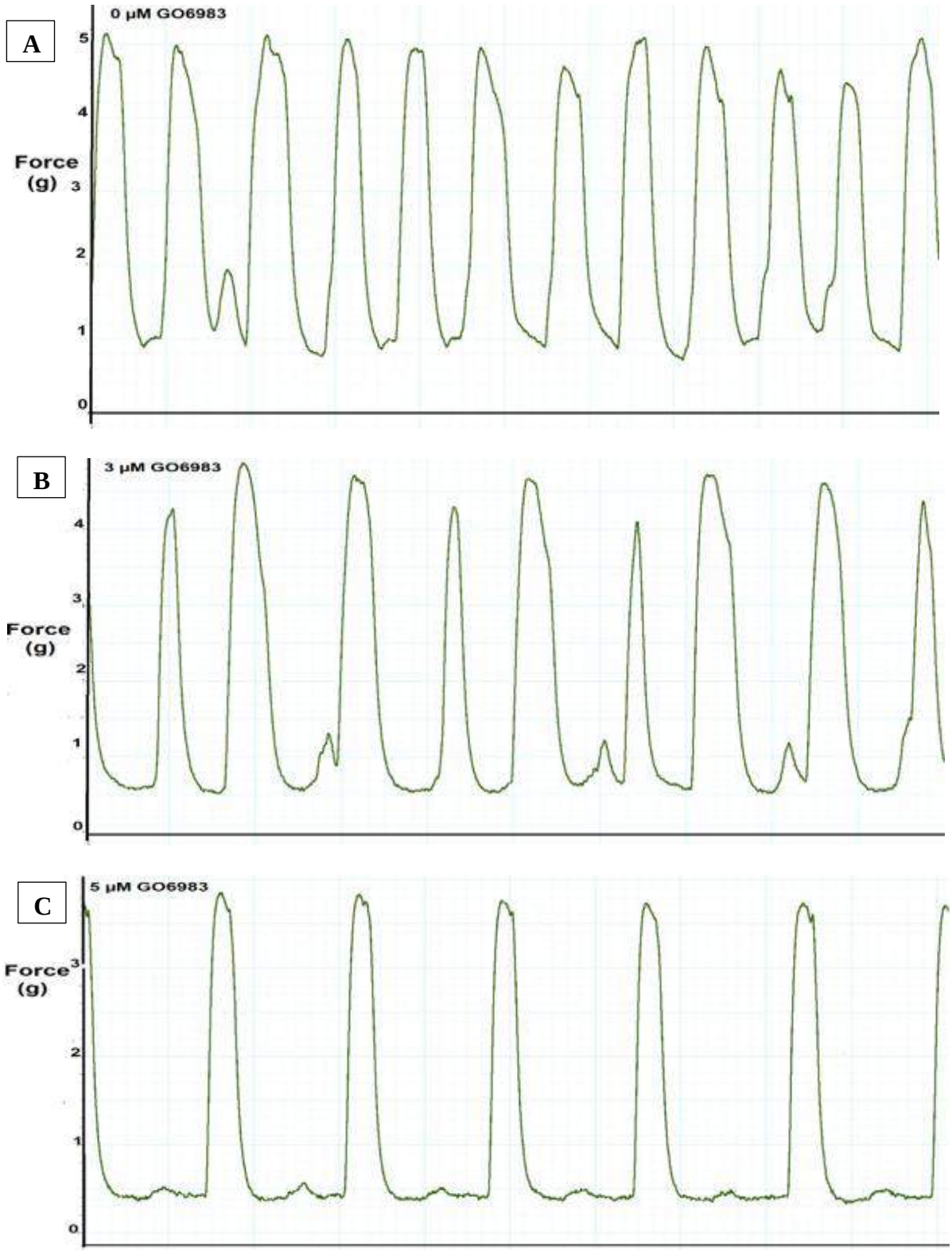

Figure 5. 7 The comparison of Go6983 treatment with concentration (A) 0 µM (krebs buffer), (B) 3 µM Go6983, (C) 5 µM Go6983 in myometrial contractile activity ex-vivo.

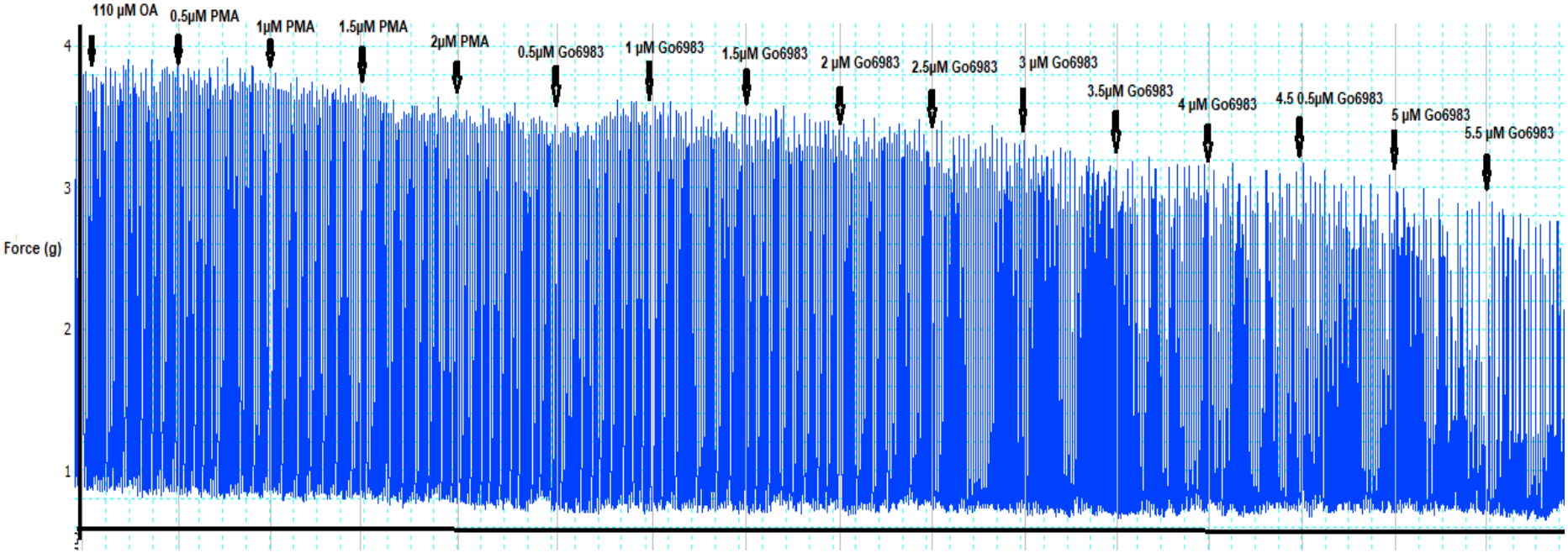

Figure 5. 8 Representative ex-vivo uterine contractility from treatment of OA in combination with PKC activator (PMA) with concentration 0.5 µM to 2µM and PKC inhibitor (Go6983) with concentration 0.5µM to 5.5 µM in combination with OA and PMA. The myometrial contraction activity was recorded in 15 minutes after each dose treatment.

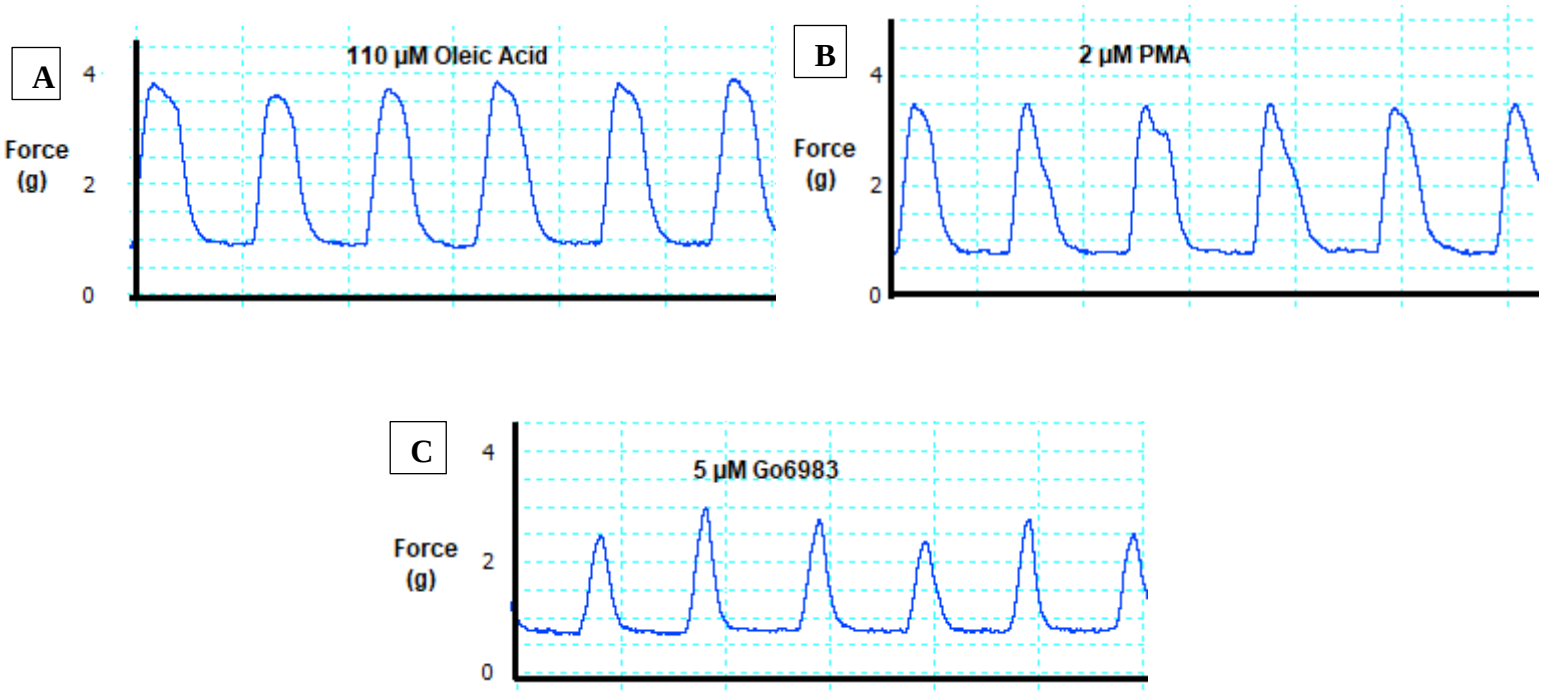

Figure 5. 9 The comparison of ex-vivo uterine contractility after exposure to (A) 110 µM OA, (B) 2 µM PKC activator (PMA) in combination with OA and (C) 5 µM Go6983 in combination with PMA and OA. The myometrial contraction activity was recorded in 15 minutes after each dose treatment.

5.3.3 Effect of PKC activator and PKC inhibitor on *ex-vivo* myometrial contractile activity

The data from **Table 5.2** identified that increasing the concentration of Go6983 had no effect on length of contraction, mean of amplitude and time to contraction peak but significantly decreased frequency, peak amplitude of contractions and mean contractile area, integral activity and maximum slope of contraction with P values of <0.01. Increasing Go6983 doses increased mean area of contraction in a dose dependent manner with IC50 = 1.78 μM (**Figure 5.10**). However, PMA in combination with OA treatment had no significant effect on myometrial contractile activity in the term pregnant rat (P-value > 0.05) (**Table 5.3**). However, from **Table 5.4** the data shows that uterine exposure to increasing concentrations of Go6983 in combination with PMA and OA significantly decreased the mean amplitude of contractions by 30% with 5 µM Go6983 (P<0.01). The area under each contraction, the integral activity and peak amplitude of contractions were significantly decreased 50% following exposure to 5 µM Go6983 when combined with PMA and OA compared to baseline (OA) (P<0.01).

GO6983 (µM)	**Frequency per 15 minutes Mean**±SEM **(P <0.01)**	**Length of Contraction (ms) Mean**±SEM **(P=0.40)**	**Mean of Amplitude (g) Mean**±SEM **(P=0.96)**	**Time to peak (ms) Mean**±SEM **(P=0.11)**	**peak of Amplitude (g) Mean**±SEM **(P<0.01)**	**Mean Area Under Contraction Mean**±SEM **(g.s) (P<0.01)**	**integral activity (g.s) Mean**±SEM **(P<0.01)**	**Maximum slope (g/s) Mean**±SEM **(P=0.88)**
0	39.5±1.5	14200±2200	2.78±0.363	4963.650±650	4.28±0.53	38.3±5.21	1520.46±263.04	3.27±0.03
0.5	40±3	12950±2650	3.29±0.06	5159±705	4.38±0.57	45.2±1.7	1713.29±303.7	3.28±0.04
1	42±0.00	11474.50±2925.5	3.11±0.8	4560±779	4.29±0.33	41.61±4.59	1480.29±459.69	3.28±0.04
1.5	37±1	14400±4200	2.9±0.5	5071±564	4.25±0.35	38.63±1.05	1428.08±0.04	3.31±0.03
2	35.50±3.5	13467±4332.5	3±0.76	5177±747.5	4.29±0.53	33.83±4.01	1186.93±23.95	3.34±0.05
2.5	37.50±6.5	13123±4476.5	3.1±0.73	4706±841	4.21±0.49	31.97±4.69	1168.39±31.93	3.30±0.05
3	34.5±4.5	12428±2672	2.72±0.43	4661.5±85.5	4.04±0.41	29.29±0.05	1010.28±130.08	3.26±0.07
3.5	33±6	12383±3417	2.80±0.53	4487.5±196.5	3.93±0.43	30.58±0.67	1012.97±205.40	3.11±0.14
4	31.50±6.5	11746.5±2753.5	3.09±0.05	4526.5±165.5	3.82±0.32	31.67±3.34	1019.125±125	3.04±0.12
4.5	28±6	12008.5±2291.5	3.15±0.09	4698±53	3.78±0.28	29.68±2.33	844.850±243.15	3.31±0.04
5	27.5.±5.5	1139±1901	3.04±0.15	4170±449	3.6±0.20	28.10±5.39	821.92±306.68	3.26±0.04

Table 5. 2 Dose response of term uterine horn strips to Go6983 (0 µM to 5 µM) . The ordinary Fit Least Square statistic was used to analyse the correlation between increasing OA dose with responding myometrial contractile activity with significance taken at the P<0.05 level. All parameters of contractile activity was measure after 15 minutes exposure to each dose.. The number of uterine strips was n=3 and values presented as means ±SEM

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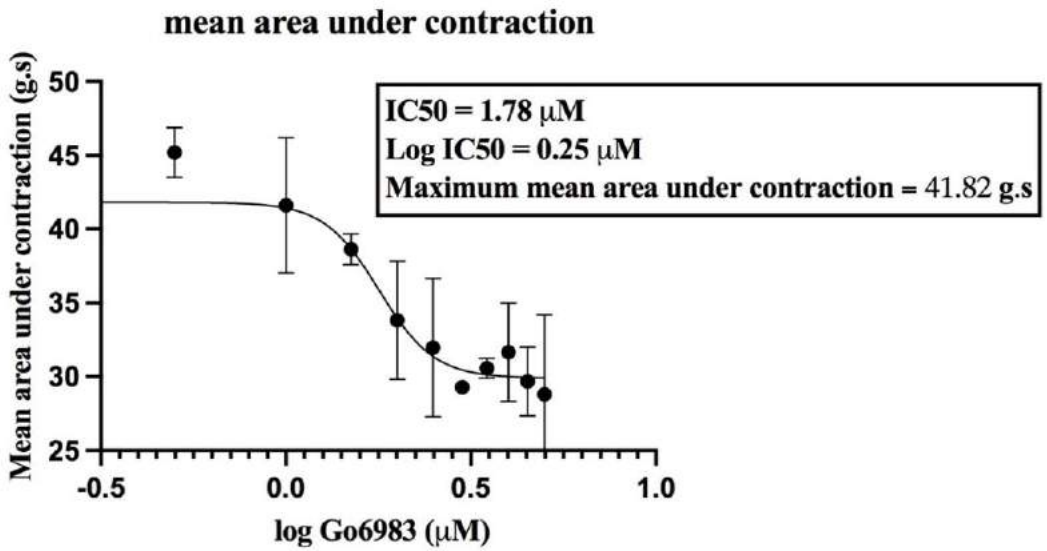

Figure 5. 10 Dose response analysis of mean area under contraction of term pregnant myometrial strips to increasing doses of Go6983 concentration (0 to 5). n=3 uterine strips. Values are means ±SEM

110 µM OA + PMA (μM)	Frequency in 15 Minutes (P= 0.275)	Length of Contraction (ms) (P = 0.696)	Mean of Amplitude (g) (P= 0.728)	Time to peak (ms) (P= 0.26)	Peak of Amplitude (g) (P=0.752)	Mean Area Under Contraction(g.s) (P=0.605)	integral activity (g.s) (P=0.706)	Maximum slope (g/s) (P=0.308)
0	22.67±2.91	25216.67±3259.27	3.01±0.62	6361.33±957.60	3.99±0.51	38.33±8.79	823.89±92.11	3.36±0.06
0.5	22±2.00	24276.67±3454.49	2.79±0.67	5097±410.34	4.047±0.51	36.57±9.23	781.99±159.11	3.09±0.25
1	26.33±1.67	21880±1062.32	2.73±0.28	5141.33±491.54	3.90±0.31	29.51±4.59	826.19±128.36	3.32±0.09
1.5	25±1.73	23580±2505.00	3.18±0.42	5600.33±813.47	4.013±0.17	33.65±7.03	622.01±285.28	3.41±0.14
2	25.33±1.20	22260±2185.56	2.94±0.27	5554.33±624.55	3.79±0.19	29.56±5.44	736.22±97.26	3.40±0.09

Table 5. 3 Dose response of term uterine horn strips to PMA (0 μM to 2 μM) in accumulation with 110 μM OA . The Repeat measured ANOVA statistic was used to analyse the correlation between increasing OA dose with responding myometrial contractile activity with significance taken at the P<0.05 level. All parameters of contractile activity was measure after 15 minutes exposure to each dose.. The number of uterine strips was n=11 and values presented as means ±SEM.

110 µM OA+2 µM PMA + Go6983 (µM)	Frequency in 15 minutes (P= 0.489)	Length of Contraction (ms) (P = 0.17)	Mean of Amplitude (g) (P= 0.060)	Time to peak (ms) (P= 0.23)	peak of Amplitude (g) (P=0.025)	Mean Area Under Contraction (g.s) (P=0.003)	integral activity (g.s) (P=0.027)	Maximum slope (g/s) (P=0.271)
0	25.33±1.20	22260±2185.57	2.94±0.27	5554.33±624.55	3.79 ± 0.19^{ab}	29.56 ± 5.44^{aced}	736.22 ± 97.25^{acde}	3.40±0.86
0.5	27.33±0.89	19423.22±826.24	2.51±0.04	5062.67±516.94	3.68 ± 0.13^{abg}	25.16 ± 1.69^{c}	685.42 ± 30.89^{a}	3.27±0.03
1	27±2.31	21716.67±1524.25	2.83±0.37	4945.33±347.83	3.83 ± 0.21^{bg}	29.35 ± 5.52^{acd}	770.80 ± 85.45^{a}	3.32±0.10
1.5	26±2.08	24386.67±1452.06	3.03±0.32	5399±652.77	3.79 ± 0.22^{bg}	30.68 ± 4.94^{ce}	7782.64 ± 88.39^{a}	3.40±0.08
2	25.67±1.45	23756.67±3140.18	2.93±0.41	5516±846.99	3.70 ± 0.20^{ah}	28.35 ± 5.61^{acb}	711.75 ± 105.92^{ac}	3.39±0.12
2.5	26±1.73	21676.67±2943.34	2.52±0.34	5095.67±787.38	3.47 ± 0.23^{abe}	23.54 ± 4.85^{db}	595.25 ± 85.51^{de}	3.32±0.10
3	26.67±1.45	21590±3912.15	2.50±0.50	5110.33±476.81	3.25 ± 0.25^{abdf}	21.99 ± 5.31^{db}	570.95 ± 109.25^{bde}	3.36±016
3.5	28.33±2.60	17483.33±1692.87	2.042±0.37	4382±93.22	3.04 ± 0.22^{cegh}	16.06 ± 3.54^{b}	440.81 ± 63.49^{b}	3.26±0.13
4	27.33±2.60	18303.33±1386.94	1.94±0.24	4649.67±396.09	2.93 ± 0.15^{dc}	15.69 ± 2.17^{abd}	423.78 ± 52.88^{cb}	3.28±0.08
4.5	27±1.53	20473.33±1876.89	2.06±0.21	5331.67±850.76	2.83 ± 0.06^{ed}	15.89 ± 1.99^{abe}	427.85 ± 557.27^{cbd}	3.38±0.09
5	25.67±2.19	20343.33±1644.12	1.83±0.33	5090.33±739.98	2.65 ± 0.08^{cdf}	13.99 ± 1.60^{abd}	360.31 ± 54.89^{e}	3.33±0.15

Table 5. 4 Dose response of term uterine horn strips to Go6983 (0 μM to 5 μM) in accumulation with 110 μM OA and PMA 2 μM . The Repeat measured ANOVA statistic was used to analyse the correlation between increasing OA dose with responding myometrial contractile activity with significance taken at the P<0.05 level. The different superscript letters are significantly different to each other within the same column. All parameters of contractile activity was measure after 15 minutes exposure to each dose. The number of uterine strips was n=11 and values presented as means ±SEM.

5.4 Discussion

The result of current study was that direct exposure of the myometrium to increasing concentrations of OA induced unsynchronized myometrial contractions and decreased the frequency of contractions, but the reverse was true in regards to the other outcome measures such as mean of amplitude, peak of amplitude and mean area under contraction which were significantly increased with increasing OA exposure. Increasing mean amplitude and peak of amplitude after increased OA accumulation might be associated with increasing conventional PKC activation as reported by Ozaki et al., (2003) who suggested that the PKC activator (phorbol 12,13-dibutylate) increases muscle tension of human pregnant myometrial strips through an increase in Ca^{2+} sensitivity related to the myometrial contraction and the level of mRNA expression of PKC β. PKC β is greater in pregnant human myometrium compared to nonpregnant suggesting a role of PKC β in inducing contractions during labour. Moreover, a study conducted by Yasuda et al., (2004) proved that the human myometrium contraction amplitude in preterm and term labour increases 2 to 2.5-fold higher when compared with nonpregnant myometrium due to the presence of PKC β. Administration of PKC β inhibitor is shown to decrease myometrial contractility up to 50%, while frequency of contractions is otherwise not influenced. In addition, the same study also illustrated that the PKC β mRNA significantly increases in the preterm and term myometrium tissue, which suggests that contraction amplitude has a positive correlation with mRNA expression of PKC β in human myometrium tissue.

Further support for PKC's involvement in myometrial contractile activity is that treatment of human pregnant myometrial cell lattices with an inhibitory peptide specific for PKC δ or with an antisense against PKC δ resulted in a significant loss of Endothelin 1-induced contraction. This therefore suggests that PKC δ plays an

important role in actin organization in human myometrium at term and has the potential role to induce myometrial contractions (Liberto et al., 2003). The theory is further supported by Kuo and Ehrlich., (2015) showing that PKC has a variety of downstream targets such as MLCK and C-kinase potentiated protein phosphatase 1 inhibitor (CPI-17), both of which increase contractions in smooth muscle (He et al., 2008). He et al., (2008) identified that knockout of smooth muscle MLCK decreased the tension, amplitude and frequency of contractions in isolated ileum dan jejenum strips. Further evidence to support that PKC δ might compromise any increase in amplitude of myometrial contractions is that PKC δ has been shown to increase the EP3 receptor that mediates vasocontraction in mesenteric arteries from type 2 diabetic rat (Ishida et al., 2012). The EP3 receptor is one of PGE2 receptor that regulates myometrial contractions during human parturition. Arulkumaran et al., (2012) identified that inhibition of EP3 decrease the amplitude contractions activity *ex-vivo* in human myometrium strips upon labour (Arulkumaran et al., 2012). The data from the current study suggest the same mechanism may be occurring where uterine exposure to increased OA concentrations during pregnancy increased amplitude and peak of amplitude through a PKC induction or activation of the EP3 receptor or through increased DAG and Ca^{2+} sensitivity related to myometrial contraction.

However, despite increased amplitude and peak amplitude of contraction, increased OA exposure induced unsynchronized contractions and decreased frequency of contraction. This might be associated with prolonged association of PKCα and PKC β which found in smooth muscle with the membrane may lead to proteolysis and release of protein kinase M into the cytosol, resulting in myosin phosphorylation and relaxation (Andrea and Walsh., 1992). Another reason OA may inhibit frequency of contractions is that OA induces gap junction disassembly through activation of PKCε, but the PKCα

inhibitor has been shown not to prevent OA-induced disassembly of gap junction in rat cardiomyocytes (Huang et al., 2004). These mechanisms could potentially occur in the uterus of pregnant rats and lead to poor contraction during established labour. The organ bath study conducted by Hag et al., (2019) support the evidence that OA accumulation decreases the frequency and amplitude of human pregnant uterine strips stimulated with oxytocin (Hag et al., 2019).

Alongside the decreased frequency of contractions, the same study also provided evidence that exposure to OA caused unsynchronized myometrial contractions that was possibly associated with PKC mediated cholesterol accumulation (Ma et al., 2006). Ma et al., (2006) reported that PKC β and PKCδ mediate cholesterol accumulation when macropinocytosis of LDL is stimulated by the PKC activator (PMA)- it activated human monocyte-derived macrophages. Further support is found where the presence of the PKC activator (PMA) together with 400 μM OA stimulated conversion of cholesterol into cholesterol esters in cooperation of glycerol being converted to trygliceride in the cultured pig coronary smooth muscle. Moreover, also identified from the same study was that the effect of the PKC activator (PMA) increased tryglyceride accumulation only in the presence of OA. These findings suggest that OA can favor long lasting effects on PKC activation within smooth muscle cells and necessary for increased lipid storage in macrophages (Moinat et al., 1990). The theory of increased lipid accumulation in the presence of OA through cooperation with PKC could possibly be occuring in uterine strips exposed to OA that exhibit decreased frequency of myometrial contractile activity, and poorly synchonized myometrial contractions as reported by Muir et al., (2016). The possible mechanism of OA alter myometrial contractile activity through PKC activation shows in **Figure 5.11.**

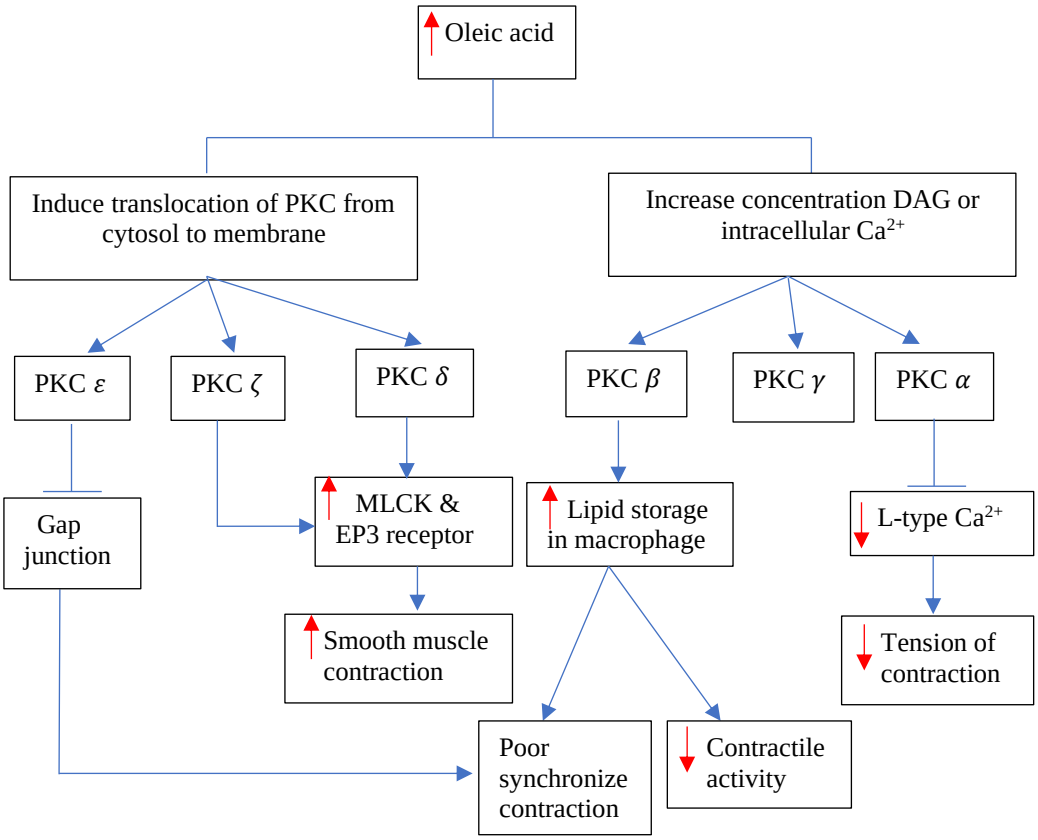

Figure 5. 11 The Possible mechanism of the effect of oleic acid exposure in the myometrial contraction activity through Protein Kinase C (PKC) activation. Increased exposure of oleic acid possibly increased concentration Diacyglycerol (DAG) and intracellular Ca^{2+} that lead to increase PKC dependent Ca^{2+} such as PKC β, PKC γ and PKC α , also potential to increase translocation of PKC from cytosol to the membrane. Increased PKC has different effect on myometrial contraction depending on the isoforms, such as PKC ε, PKC β, PKC α potential to decreased contractile activity and caused poor synchronize contraction, while PKC ζ and PKC δ potential to improve contraction through Myosin Light chain Kinase (MLCK).

Furthermore, This experiment then determined whether OA exerts its effects via the PKC pathway. The current study provides evidence that Go6983 exposure significantly decreases frequency, amplitude and peak amplitude of myometrial contractions and furthermore that Go6983 in combination with PMA and OA significantly reversed the pattern of myometrial contraction after OA exposure alone. This showed increased frequency of contractions but decreased mean contraction area, peak amplitude and integral activity of contractions. These suggests that Go6983 exerts its effects through inhibiiton of PKC such as PKC α, PKCβ and PKCδ in myometrial strips as reported by (Ozaki et al., 2003). Ozaki et al., (2003) also identified that G06983

induced a marked inhibition of phorbol ester which acts as PKC activator that can increase high K^+ induced contractions in human strips of myometrium. Further evidence illustrating the inhibitory effect of PKC through pretreatment with Go6983 is in rat aorta where it significantly decreased high K^+ induced contractions after exposure to urotension II (Tasaki *et al.,* 2004).

However, combined exposure to PMA with OA had no significant effect on the pregnant rat myometrial contractions. Our finding that PMA did not have any impact on myometrial contractile activity contrasts with Itoh and Lederis (1987) that identified PMA induced a slow and progressive increase in tension of rat aorta strips in the presence of extracellular Ca^{2+} and still induced a small but sustained contraction in the absence of extracellular Ca2+. However, a study conducted by Chardonnens et al., (1990) proved that PMA induced PKC activation for various periods of time on the membrane of aortic smooth muscle cells decreased cytosolic PKC activation. Kim et al., (1996) identified that there was no effect of phorbol esters on the Ca^{2+} induced contraction in rat myometrium that suggests it is distinct from other types of smooth muscles cells, as the rat myometrium does not have a PKC-mediated Ca^{2+} sensitizing process to activate contractile activity. Another possible reason PMA exposure in cooperation with OA did not significantly effect myometrial contractions is that OA has already induced the PKC activator to decrease contractile frequency and increase the amplitude of myometrial contractions and as a result PMA cannot increase the PKC activity any further.

5.5 The strength and limitation of study

The current study proved that OA affect myometrium contraction through PKC in dose dependent manner in term pregnancy in the rat. From this study provide evidence

that direct exposure to OA leads to poorly synchronized myometrial contractions and decreased frequency of contractions but increased mean and peak amplitude and time to peak in dose a dependent manner in the myometrium from term pregnant rats. The poorly synchronized myometrial contractions and decreased frequency of contractions after OA exposure suggest that OA may cause their effects through PKC activation, especially PKCα . This theory is supported by Kim et al., (2003) who observed that PKC is expressed in rat myometrium, but the predominant isofom detected by western blot is PKCα and its expression gradually increases during gestation and reaches maximal expression at day 21 of gestation. Meanwhile, other dominant PKC isoforms such as PKC δ and PKCε decrease during gestation reaching minimal expression during late pregnancy. The same study also prove that exposure to a PKCα activator inhibits the tension induced by high K+ in the rat myometrium. Kim et al., (2003) also speculated that PKCα activation may activate membrane Ca^{2+} pumps and inhibit the L-type Ca^{2+} channel resulting in decreased Ca^{2+} and muscle tension. However, the current study did not investigate PKC expression after OA exposure in the pregnant rat myometrium nor PKC activation. Therefore, western blot analysis might be helpful for future studies to investigate the effect of OA exposure on myometrial rat tissue or in the isolated myometrial rat cells during late of gestation, do determine the PKC isoform that OA may be activating and involved in regulating myometrial contractile activity.

Another limitation of this study was that the investigation of the effects of PKC activator and PKC inhibitor on uterine contraction was done in parallel, therefore it was not clear whether the PKC activator (PMA) had an effect on the PKC inhibitor (Go6983). However, in a separate investigation the author conducted an experiment to examine the individual effect of PKC inhibitor Go6983 towards uterine contractions, a significant decrease in the peak amplitude and frequency of uterine contractions were evident. This

suggests that Go6983 inhibits conventional PKC and PKC δ as mentioned by Ozaki et al (2003) and that Go6983 particularly inhibits PKC δ, PKCβ and PKCα in human myometrial tissue and inhibit PKCδ in PMA induced PKC activator in human teratocarcinoma cells (Wen et al., 2003).

6. General discussion

The mechanism underlying prolonged and dysfunctional labour in maternal obesity is still unclear. Our group research established that that maternal obesity decreases expression of the protein Connexin-43 and increases plasma progesterone concentrations at term pregnancy in the rat (Muir et al., 2016). Furthermore, it has been identified that chronic exposure to a HFHC diet significantly decreases proportion of plasma and liver omega-3 and omega-6 but increases OA in the pregnant rat. Interestingly, only omega-3 and DPA decrease significantly in the uterus of rats fed a HFHC diet (Muir et al., 2016). A study also reported that changing diet from a HFHC diet to control diet at conception reverses the fatty acid profile in the plasma, liver and uterus (Muir et al., 2018). The significant increase in total omega-3 PUFA concentrations in the uterus after changing from a HFHC to control diet at conception has the potential to increase prostaglandin production and alters expression of key proteins involved in myometrial contractile activity. Thus, the aim of the thesis was to investigate the potential mechanisms behind dysfunctional and prolonged labour in HFHC fed rats.

The first experiment evaluated the effect of chronic exposure to a HFHC diet on the histology of the uterus from term or labouring rats. The important finding was that the HFHC diet has a significantly higher number and mean area of vacuole within the myometrium compared with uterine tissue from control labour rats. However, since the tissue was fixed in ethanol, it might have caused lipid loss during fixation and dehydration process. Morgan and Huber (1967) provide evidence that fixing tissue in 4% formalin followed by alcohol lost 47% of the lipid from pig lung tissue. From the same study and Leist et al., (1986) reported that fixing the tissue with 3% glutaraldehyde can prevent such lipid losses. For that reason, only myometrial vacuoles are assumed to be areas where fat accumulated since Morgan and Huber (1967) also mentioned that higher lipid loss may be due to the much higher content of saturated fatty acids. However, the greater number and area of vacuole in HFHC rat tissue might

represent the HFHC diet increasing the fat content within uterine tissue and be the potential mechanism behind dysfunctional labour with maternal obesity. This is based on a recent study that reported that knocking out the LXR gene, which regulates cholesterol homeostasis increases lipid accumulation especially cholesteryl esters in the myometrium causing muscular defects in the uterine smooth muscle contractile activity in the mouse (Mouzat et al., 2007). High fat diet possibly increased lipid droplets in muscle and increased intramyocellular lipid in smooth muscle of the uterus resulting in reduced contractile activity. This is supported by Eshima et al., (2020) that proved the evidence that high fat diet has been shown to significantly increase intramyocellular lipid compared to that of a low fat diet in digitorum longus muscles in the male rat that was associated with degree of impaired muscle contractile force and low peak Ca^{2+} level. The same mechanism potentially occurs in the smooth muscle uterus of pregnant rat that fed high fat diet. Furthermore, Gam et al., (2017) provide further evidence that maternal obesity reduced myocyte density with increased triglyceride content in myometrium which could lead to poor uterine contraction in the pregnant women. All the experimental findings stated above explains how a high fat diet could increase lipid accumulation and associated with smaller muscle content that may lead to reduce contractile activity in smooth muscle tissue.

A number of studies have also highlighted that fatty acids contribute to labour outcomes in both humans and animals (Cheng et al., 2013; Cheng et al., 2001; Kirkup et al., 2010; Roman et al., 2006). One animal study supporting this theory is one identifying that high linoleic and OA consumption post-conception enhances Prostaglandin type 2 and likely increase myometrium contractility in the onset of labour (Cheng et al., 2015; Elmes et al., 2005). However, other studies have reported that omega-3 PUFA decreases preterm labour through inhibition of pro-inflammation agents in the mouse uterus and prostaglandin type 2 suppression in the bovine uterus (Coyne et al., 2008; Yamashita et al., 2013). Meanwhile, the omega-6

PUFA especially arachidonic acid is a substrate for COX-2 synthesis that act as precursor for prostaglandin type 2 synthesis (Kirkup et al., 2010). However, the study investigating the effect of HFHC diet during pregnancy on fatty acid levels in the plasma, liver, placenta and uterus is still limited. Accumulation of fatty acids within the myometrium could potentially affect contractile activity of the myometrium . Therefore, the next experiment was set up to analyse the effect of the obesity inducing HFHC diet on the fatty acid composition (%) of the plasma, liver, uterus and placenta. Critically, the study also observed the effect of switching diet from control to HFHC and HFHC to control at conception.

The evidence of the current study was that exposure to the HFHC diet post-conception significantly altered the fatty acid composition of the plasma, liver, uterus and placenta. The omega-6, omega-3 PUFAs in both plasma and liver were significantly decreased, whereas the saturated fatty acid and OA was significantly increased in the HFHC group compared to controls. Interestingly, the study established that switching diet during pregnancy reversed fatty acid profile in the plasma and liver. This study suggests that variations in circulation and liver fatty acids in rats that consume an obesogenic diet before pregnancy can be changed by switching to control diet during pregnancy.

One interesting result in this study was that only total omega-3 PUFA was significantly lower in uterus HFHC group compared with control group. This study suggests that the uterus is more resistant to changes in fatty acids, and another explanation might be some of the fatty acids are utilised and necessary for labour. Evidence from the current study suggests that chronic exposure to a HFHC diet during pregnancy significantly increases DGLA concentrations in the pregnant rat placenta. Increased DGLA levels in the HFHC group might contribute to prolonged labour through proinflammation cytokine inhibition since Dooper et al., (2003) provides evidence that 100 µM DGLA supplementation to isolated human peripheral blood mononuclear cells for 48 hours significantly decreased kinetics of IL-10 and

TNFα by 60%. If the inhibition of IL-10 and TNFα by DGLA occurs in placenta, this can be a potential mechanism of dysfunctional labour in maternal obesity since many studies provide evidence that induced TNF α to myometrium tissues and cells increase myometrial contractions through increased production of prostaglandin type-2 in human and animal study (Sivarajasingam et al., 2016). Further evidence is also provided to support this theory where adding 20 μM DGLA to IL-1 stimulated synovial cells decrease concentration of PGE_2 by up to 70% (Baker et al., 1989). Fischer et al., (2020) proved the evidence that PGE_2 has the highest affinity to binds with EP-3 receptor subtype compared than other EP receptor (EP_3>EP_4>EP_2>EP_1) which enhance in late gestation and at term labour *in-vivo* study in human uterus. In addition, EP-3 receptor mRNA is highly express in the mouse uterus (Sugimoto et al., 1992). An immunohistochemistry analysis conducted by Grigsby et al., (2006) found that all EP receptors localize in placenta and another study proved that protein EP1, EP3 receptor reported increase in labour pregnant human trophoblast and syncytiotrophoblast cells (Unlugedik, 2009).

The establish mechanism behind prolonged and dysfunctional labour in maternal obesity is related to increased progesterone concentrations at term pregnancy in the rat (Muir et al., 2016). Support for this mechanistic theory is the fact that human labour can occur in the presence of elevated oestrogen concentrations in circulation but where progesterone levels increase slowly in the myometrium (Nadeem et al., 2016). These findings suggest that there is a crucial mechanism associated with oestrogen synthesis that may be associated with unsynchronised contractions with maternal obesity. Garnier et al., ((2015) reported that activation of PPAR-γ increase receptor mRNA, mRNA and protein of Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) in extravillious trophoblast cell line and blocking of VEGF signalling with VEGFR tyrosine associated with increased plasma progesterone levels leading to a stimulation of myometrial contractions in mice (Wada et al.,

2013). Furthermore, a study conducted by Fan et al. (2005) proved that inhibition of PPAR γ activity could suppress Nf-kB transactivation and inhibit aromatase activity, a crucial enzyme that controls oestrogen synthesis via Nf-kB in human ovarian granulosa cells. Therefore, the next step was to establish whether the same is true within the placenta through analysis of the effect of a HFHC diet on protein expression of placental PPARs and aromatase. Thus, Western Blot analysis was used to investigate the potential impact of HFHC diet to suppress myometrium contractility through PPARs and aromatase expression in rat placenta.

This current study provides evidence that protein expression of PPARγ increased significantly in the placenta from HFHC compared to the control group. PPARγ is a nuclear receptor that plays a pro-inflammatory role and cytokine inhibitor that could suppress uterine contractions (Schaiff et al., 2006). Surprisingly, it has been observed that although the placental omega-6 LA and DGLA significantly increased in the HFHC group, PPARγ protein level still remained higher in the HFHC groups compared to controls. The potential reason for this might be due to the fact that the fatty acids were obtained and analysed as a percentage of total fatty acids, so it might not represent the actual amount of fatty acids within the tissues. The other potential theory is that maternal obesity leads to lipid accumulation, and insulin-resistance thus increasing activation of PPAR γ to suppress COX-2 synthesis and stimulate myometrial relaxation. This is in line with Sikder et al. (2018) who shows chronic exposure to a high fat diet mediates the activation and translocation of PPARγ from nuclear to cytosolic and increase the mRNA and protein expression of PPARγ induced by intracellular lipid and lipid accumulation in the murine heart. Thus, the author speculates that the same mechanism occurs in the placenta. Many studies also support this theory as PPAR γ activation is important to maintain uterine quiescence by suppressing COX-2 synthesis and proinflammation cytokine activation in human placenta (Borel et al.,2008). Moreover, some studies in human cells and mice reported that increasing activity of PPAR and upregulating mRNA and protein expression

of PPAR increase VEGF level in macrophages, vascular smooth muscle cells and the corpus luteum and therefore, this condition is associated with increasing progesterone secretion in plasma and prolonged labour (Fraser and Wulff, 2003; Froment et al., 2006; Wada et al., 2013; Yamakawa et al., 2000).

A study reported that activation of PPAR γ decreases aromatase activity in the human ovarian cells (Fan et al., 2005; Margalit et al., 2012). However, the current study illustrated that even though protein expression of PPAR γ was higher in the HFHC compared than control group, but the protein expression of aromatase was not significantly different between HFHC and control group. Subbaramaiah and colleagues (2012) and Cruz et al., (2005) reported that PPARγ inhibits aromatase activity through decreased level of protein COX-2 and PGE_2 in human and mice preadipocyte mammary gland cells. However, the established study mentioned that protein expression of COX-2 in the uterus and plasma PGE_2 concentration was not significantly different between HFHC and control group at term of pregnant rat. This suggests that PPAR γ possibly had no role in altering the COX-2 and PGE2 signalling pathway, thus indicating that an increase in PPAR γ concentration would not inhibit the expression of aromatase in the placenta.

Further interesting result in this current study was switched diet from HFHC to control diet during pregnancy significantly increased omega-3 (DHA and EPA), arachidonic acid, and OA in the plasma and liver. Importantly it has been suggested that supplementation with DHA, EPA, LA and OA alter prostaglandin synthesis (Cheng et al., 2015; Coyne et al., 2008; Elmes et al., 2005; Kirkup et al., 2010; Roman et al., 2006). EPA acts as a competitive inhibitor and displaces arachidonic acid (AA), a substrate for COX-2 synthesis, resulting in increased prostaglandin type-3 synthesis and decreasing Prostaglandin type-2 synthesis (Allen and Harris, 2001). Therefore, the different amount or ratio of omega-6 and omega-3 FAs may alter the amount and type of prostaglandins being synthesised, resulting in different labour

outcomes. Moreover, Cheng et al. (2015) established the theory that OA increased maternal endometrium PGE_2 production but attenuated $PGF_{2\alpha}$ leading to a doubling in proportion of PGE_2 : $PGF_{2\alpha}$ which suggests that OA might contribute to both stimulating and inhibiting parturition. As a result we investigated whether there was any correlation between fatty acids in the uterus and maternal plasma prostaglandin concentrations. However, we confirmed that there was a no correlation between uterine fatty acid and plasma prostaglandin type-2. At term labour, any PGE_2 and $PGF_{2\alpha}$ synthesised will quickly be converted into their respective inactive metabolite PGEM and PGFM and potentially be the reason behind no significant differences observed in PGE_2 and $PGF_{2\alpha}$ production in the control and HFHC groups. This result is consistent with the study conducted by Green et (1974) that identified no correlation of plasma $PGF_{2\alpha}$ with stage of labour, while the level of plasma PGFM increased significantly from the first trimester right up until term labour in pregnant women. Furthermore, the level of plasma PGFM mirrored the $PGF_{2\alpha}$ extracted from the placenta during late gestation in the pregnant cat (Siemieniuch et al., 2014). Thus, the level of plasma prostaglandin metabolites would be more robust and consistent for indirect analysis of plasma PGE_2 and $PGF_{2\alpha}$ since PGE_2 and $PGF_{2\alpha}$ will be converted to PGFM and PGEM at the end of pregnancy.

Furthermore, our finding illustrated that plasma and liver proportion of omega-3 and omega-6 decreased significantly after HFHC treatment but only omega-3 proportion decreased significantly in the uterus. Muir et al. (2016) proved that a HFHC diet decreases omega-3 in the rat uterus and inhibits expression of key contractile associated protein (CAPs) that have the potential to supress myometrial contractions. This suggests that the ratio of omega-3 and omega-6 PUFAS might play an important role in myometrial contractions. The next experiment therefore evaluated whether improving omega-3 PUFA status via diet could potentially affect myometrial contraction in the pregnant rat uterus. Thus, the next experiment was to observe the effect of different ratios of LA: ALA at two different total fat levels on the fatty acid

composition of the plasma and uterus. The interesting result of this study was that it was similar to the previous experiment in chapter 3 that illustrated the plasma was more responsive to the changes in fatty acids levels compared to the uterus. In addition, the high fat diet significantly contributed to decreases in both omega-3 and omega-6 PUFAs in the plasma. This is not surprising that the plasma is more responsive to dietary fatty acids because the plasma is sensitive to short term (days until months) fluctuations in fatty acid intake and the plasma plays a critical role in carrying nutrients into tissues pool and away from the tissues pool for utilisation by target tissues (Shibutami et al., 2021). Moreover, there was an interaction between diet and the level of fat that significantly affected AA and EPA where the high LA high fat diet significantly decreased AA, while high ALA followed with high level of fat significantly decreased EPA. This suggests that higher total fat levels associated with maternal obesity might have a negative impact in desaturation and elongation process as mentioned by Wang *et al.,* (2006) that stated that mice fed a high saturated fatty acid diet reduces hepatic Fads1 and Fads2 gene expression as well as Elovl5 expression and activation.

Change of fatty acid profile in the uterus has the potential to affect myometrial contractile activity via contractile associated protein (CAP) expression (Muir et al., 2016). Importantly, omega-3 and omega-6 fatty acids can decrease or increase the membrane fluidity, which then modifies the ability of proteins to form receptor complexes and migrate into the membrane field (Murphy, 1990). Prasad et al., (2010) reported that omega-3 induce higher membrane fluidity compared to omega-6 and potentially affects signal transduction including several transport proteins which could help to transport omega-3 from membrane to intracellular resulting in modification of signalling in the intracellular. Meanwhile review study conducted by Baastaarse et al., (1997) proved that increased cholesterol can decrease membrane fluidity and vice versa in cell and tissues study and therefore, could affects gap junction assembly, Ca^{2+} channel and intracellular signal in human and rat cardiomyocytes. At

higher cholesterol content, gap junction formation is shown to has negative affect or even has lower level of gap junction compared than control group in hepatoma cells. Therefore, we investigated the effect of different ratios of LA and ALA fed at 2 different fat levels on uterine expression of CAPs. The result of the current study identified that high ALA diet significantly increased CX-43 protein in the uterus at term pregnancy. ALA possibly induces a CX-43 response by interacting with membrane receptors such as G Protein receptor that then activate protein kinase, which is essential to modify opening of gap junction channel and/or modify assembly of connexin (Puebla et al., 2017). Increased connexin will increase communication between myometrial cells and help synchronise myometrium smooth muscle contractions during labour (Albrecht et al., 1996).

We also provided nice evidence that a high omega-3 ALA diet at conception increased Cav-1 protein expression in the uterus. Many studies provide evidence that higher mRNA and protein Cav-1 could inhibit the redistribution of essential proteins that mediate smooth muscle contractility such as protein rhoA, PKC-α and ROK that are all crucial to inducing Ca^{+} release from the sarcoplasmic reticulum making cells more excitable (Lee et al., 2001b; Taggart, 2001; Taggart and Wray, 1998). Some studies reported that Ca^{2+} depletion in the sarcoplasmic reticulum increases myometrium contraction significantly at term labour in both humans and animal models (Matthew et al., 2004; Noble et al., 2006). Therefore, this study suggests that improving omega- 3 dietary status may potentially increase myometrial contractions during labour through increased expression of the CAPs including protein expression of CX-43 and Cav-1.

Overwhelming evidence from this study was that different proportions of LA (9:1) and ALA (1:1.5) did not affect COX-2 protein expression, but a high total fat level did significantly increase COX-2 protein expression. It is suggested that a high ALA diet increases COX-2 protein expression only if fed at a high level. The evidence from the current study is supported

by Wang et al., (2016) who identified that exposure to a high fat diet induced COX-2 mRNA and protein expression through modulation of Insulin like Growth Factor (IGF) and IL-6 as a pro-inflammation factor in animal study (Hamzawy et al., 2015; Wang et al., 2016). The other important evidence from this current study was explained that diet and fat significantly affect protein PPARα expression. The (9:1 36%) group had 40% higher PPAR α protein expression compared to the (9:1 18%) group. The evidence of higher PPAR α protein expression in the higher total fat diet also explains the important regulation of fat in protein expression, especially in regard to PPARα. Further evidence of this is reported in studies that increased de novo lipogenesis has a contribution to fat accumulation in obesity, and PPARα can suppress the expression of acetyl-CoA carboxylase (ACC) which inhibits de novo fatty acid synthesis, further decreasing intracellular fatty acid levels available for triglyceride synthesis (Yoon, 2009). Moreover, it has been reported that PPAR α contributes to suppressing COX-2 synthesis (Grau et al., 2006; Coyne, Kenny, 2008). The potential mechanism of supressing COX-2 though PPARα is that increased protein expression of PPARα is associated with decreased concentrations of IL-1β, a pro-inflammatory agent that plays a critical role in stimulating arachidonic acid release and increases the synthesis of COX-2. Therefore, inhibition of IL-1β through PPARα pathways could possibly increase relaxation of the myometrium in a human (Rauk and Chiao, 2000). This theory is supported by Dong et al. (2013) who illustrated a negative correlation between PPAR-α and IL-1β on mRNA and protein expression in human myometrium. Increased PPARα protein might play an essential role in maintaining pregnancy by inhibiting myometrial contractions through suppression of IL-1β. However, IL-1β might be needed to be observed in future studies to prove this theory.

The important results from chapters 3 and 4 was that OA levels in the plasma and liver were affected by diet, also the plasma $PGF_2\alpha$ was significantly increased if the proportion of OA in the uterus increased. This suggest that OA treatment might affect myometrial

contractions, therefore the organ bath study was setup to investigate the effect of OA supplementation on rat uterine contractions *in-vivo*. Our findings were that OA effects uterine contractions but that it might be dependent on the activation of a specific PKC isoform since addition of the PKC inhibitor (G06983) significantly increased frequency but decreased peak amplitude, peak area under contraction and integral activity of the uterine contractions. This suggests that Go6983 has the opposite effect to OA. Studies have also identified that Go6983 preferentially inhibits PKCα, PKCβ and PKCδ, but is effective against PKCδ at a 500nM concentration (Anastassiadis et al., 2011; Ozaki et al., 2003). Thus, this data suggests that OA alters myometrial contractions through activation of PKC.

Administration of OA to uterine strips of pregnant rats increased both mean and peak amplitude of myometrial contractions and potentially working through altered activity of PKCδ since Ishida et al. (2012) reported that enhancement of EP-3 receptor- mediated vasocontraction is inhibited by rotterlin, the specific PKC δ inhibitor in diabetic arteries rat . It suggests that PKCδ pathway might contribute to increase EP3 receptors and modulate mesenteric arteries contraction in diabetic rats. The same mechanism possibly occurs in the uterus after administration with OA. Moreover, another plausible of mechanism related increased amplitude after OA administration was that OA potential to induce PKCβ wich stimulate Ca^{2+} sensitive contractions and increase the amplitude of human myometrial contractions during pregnancy (Yasuda et al., 2007).

The increased amplitude and peak amplitude of contractions could also be related to PKC δ or PKC ζ because Kim et al., (1999) reported that PKC δ and PKC ζ protein expression remained stable during the later days of gestation and immunohistochemical analysis confirmed that the expression of PKC δ and PKC ζ found in both circular and longitudinal smooth muscle layers of the rat uterus. Liberto et al., (2003) illustrated that treatment of myometrial cell with specific PKC ζ inhibitor resulted in significant loss of endothelin-1

induced contraction that has important role in actin organization and potential to stimulate myometrium during parturition. In addition, many studies also proved that Endothelin-1 has important role to increase Ca^{2+} sensitization and activate MAPK that prominent for contraction (Wynne et al., 2009; Liberto et al., 2003).

Despite increased peak and mean amplitude of contractions following exposure to OA there was a significant decrease in the frequency of contractions that exhibited de-synchronized contractions. Poorly synchronized myometrial contractions after exposure to increasing concentrations of OA could be related to PKCε since Huang et al., (2006) provides evidence that OA treatment induced PKCε activation that increased gap junction disassembly and inhibited contraction of rat cardiomyocytes. The role of OA inducing PKCε activation may possibly occur in the uterus and contribute to uncoordinated and dysfunctional contractions during labour, particularly since Muir et al. (2016) identified increased protein expression of phosphorylated CX-43 in the uterus of obese term and labouring rats.. In addition, Kim et al., (2003) identified that PKCα increases gradually in the rat myometrium during gestation and reaches maximal expression at day 21 of gestation (term labour) and this PKC isoform has the potential to inhibit L-type Ca^{2+} channel resulting in decreased muscle tension. Another plausible cause of unsynchronized contractions following OA treatment is OA can favor long lasting effects on PKCβ activation within smooth muscle cells necessary for increased lipid storage in pig coronary smooth muscles and human macrophages (Ma et al., 2006; Moinat et al., 1990). Increased lipid storage in the presence of OA through cooperation with PKCβ could possibly occur in the uterine strips exposed to OA that caused poorly synchronized contractions.

Overall, this study has illustrated that diet, especially the level of fat and fatty acids including omega-3 and omega-6 PUFA and omega-9 OA composition contributes to significant changes in the fatty acids within the uterus and placenta and has the potential to

affect contractions during labour through proteins that regulate myometrial contractions at term labour such as PPARs and CAPs and potential to activate PKC (**Figure 6.1**). Interaction between high omega-3 and omega-6 diet fed at different total fat level affected CAPs and PPARs, where PPARs contribute to regulate lipid homeostasis and have an interaction with pro-inflammatory cytokines such as Nf-kB, IL-β, TNFα, IL-6, IL-8. High fat diet increases PPARγ protein in the placenta and an interaction between high fat and high omega-6 diet significantly increased PPARα protein in the uterus. Increasing PPARγ and PPARα protein expression could potentially inhibit Nf-kb that could suppress prostaglandin type 2 production leading to decreasing uterine contractions further. Interestingly, increasing the dietary omega-3 consumption during pregnancy was accompanied by increased COX-2 protein expression, a substrate for prostaglandin type-2 production where many studies provide evidence that increasing COX-2 is associated with increased myometrial contractions in both human and rat studies (Slater et al., 1999; Engstrom, 2001). Furthermore, exposure to a high omega-3 PUFA diet also increased protein expression of myometrial Cx-43 and Cav-1 at term labour in the rat. Increased protein expression of Cx-43 associated with increased communication between cells within the uterus that regulate synchronization of contractions, while Cav-1 is essential for caveolae formation and play an important role in Ca^{2+} handling since Daniel et al., (2009) reported that knockout of caveolae where Cav-1 being one of crucial structural component will reduce Ca^{2+} extracellular and decrease the ability of open L-type Ca^{2+} channel to supply some calcium for contraction in pig intestinal. Brainard et al., (2005) also identified a correlation between disruption of caveolae and altered calcium signalling, it led to inhibition of intracellular Ca sensitive Maxi-K channels and decreased human smooth muscle force. Therefore, it suggests that decreased expression of CX-43 and Cav-1 following low dietary omega-3 exposure could suppress contractile activity or cause uncoordinated myometrial contractions at term labour in the rat. In addition, omega-9 OA affected myometrium

contractions through activation of PKC. This study illustrated that exposure to high OA levels significantly increased amplitude of uterine contractions but decreased their frequency through PKC activation.

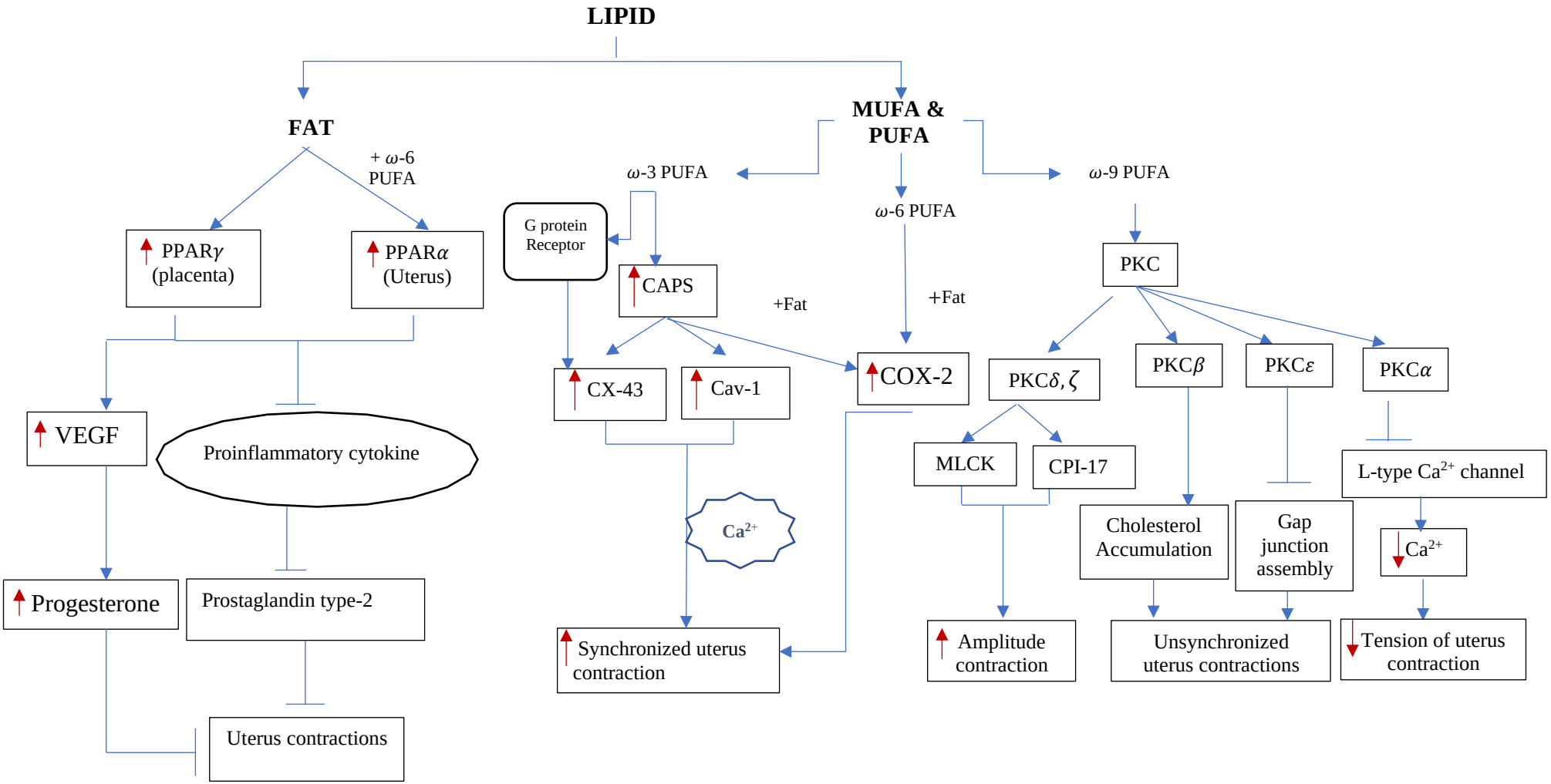

Figure 6. 1 The Possible effect of high fat and fatty acids on myometrial contraction. MUFA : Monounsaturated fatty acid, PUFA : Polyunsaturated fatty acid, PKC : Protein Kinase C, VEGF: Vascular endothelial growth factor, CX-43 : Connexin-43, COX-2 : Cyclooxygenase-2, Cav-1: Caveolin-1, PPAR: Peroxisome proliferator-activated receptor, MLCK : Myosin light chain kinase, CPI-17 : Phosphorylation-dependent inhibitor of myosin phosphatase

It is important to note the current study does have limitations that need to be considered. The histological analysis is not ideal for identification of lipid within the uterine tissue. The Oil red O staining is ideal for observing lipid accumulation in the tissues, but the uterine tissue had been processed in ethanol before being fixed in formalin, therefore, the lipid may have dissolved out of the tissue, as a result the lipid can only can only be analysed crudely from the number of empty spaces evident within the tissue. Moreover, fatty acid concentrations in the uterus were not expressed as absolute fatty acid concentrations and only represent the percentage of total fatty acids . Another limitation of this study was that the plasma analysis of $PGF_{2\alpha}$ and PGE_2 in pregnant rat during term of labour was not via the more stable metabolites PGFM and PGEM since It was identified by Smith (2001) that oestrogen upregulates COX-2 expression in maternal endometrial tissue and is associated with increased PGDH expression and leading to increased $PGF_{2\alpha}$ output to PGFM in late-onset labour. Therefore, the metabolites PGFM and PGEM would be more appropriate for observing prostaglandin concentrations rather than $PGF_{2\alpha}$ and PGE_2 at the onset of labour.

Moreover, another limitation within the thesis was the investigation of the effects of the PKC activator and PKC inhibitor on uterine contractions, it was done in parallel, therefore it was not clear whether the PKC activator (PMA) had an effect on the PKC inhibitor (Go6983). The PKC activator in combination with accumulating OA treatment did not affect uterine contractions. We suggest that PKC is already activated by OA treatment, and as a result, PMA could not increase contractile activity any further. However, in a separate investigation the author conducted an experiment to examine the individual effect of PKC inhibitor Go6983 towards uterine contractions, a significant decrease in the peak amplitude and frequency of uterine contractions were evident. Moreover, PKC inhibitor (Go6983) in combination with PMA and OA significantly increased frequency but decreased peak amplitude, peak area under

contraction and integral activity of contractions. This suggests that Go6983 has the opposite effect to OA. Some studies identified that Go6983 preferentially inhibits PKC α, PKCβ and PKCδ, but is effective against PKCδ at a 500nM concentration (Anastassiadis et al., 2011; Ozaki et al., 2003). Thus, this data suggests that OA altered myometrial contractions through activation of PKC, especially PKC α, PKCβ and PKCδ.

Future Research

Due to time and tissue restrictions, not every research question could be explored within this project. Therefore, the suggestion for future research is discussed below.

The current histology protocol had not stained and counted actual lipid accumulation in the uterine tissues and the fixing protocol in future should be improved. The tissue could be fixed in osmium tetroxide to preserve the lipids as published by Carriel et al. (2017). The oil red stain can then be used to stain and calculate the lipid content after chronic exposure to the HFHC diet.

Our research identified that aromatase was not significantly different between the HFHC and control groups and might suggest that other enzymes such as Cyp17 (P450c17α) and 17α hydroxylase enzymes might need to be investigated as potential enzymes that alter oestrogen synthesis at term labour.

Moreover, PKCα, PKCδ, PKC ζ and PKCβ might be activated by OA and affect myometrium contraction during parturition. The organ bath study would have been scientifically better to treat myometrial strips with the OA followed with PKC activator and OA followed with PKC inhibitor which would help elucidate whether OA exerts its effects via the PKC pathway. Moreover, future western blot analysis would be useful to investigate the effect of OA exposure on PKC expression or PKC activation in myometrial rat tissue or in the isolated myometrial rat cells during late gestation, to determine the PKC isoform that OA may be activating and involved in regulating myometrial contractile activity.

Clinical Impact of The Study

The mechanism behind prolonged and dysfunctional labour with maternal obesity has not been completely elucidated. Interestingly, this study has revealed that it is the diet associated with obesity rather than obesity itself that may be causing prolonged and dysfunctional labour through alterations in protein signalling that have a detrimental impact on myometrial contractions. Through this research, the health care team such as obstetricians, gynaecologists, nutritionists and dieticians would have new insights for potential dietary or drug treatments to reduce the risk of prolonged and dysfunctional labour in obese pregnancies. A diet high in omega-3 PUFA would be beneficial for maternal obesity, since we provide evidence that a high omega-3 diet has the potential to improve myometrial contractile activity through increased protein expression of CX-43 and CAV-1 in the term and labouring uterus. Therefore, consuming a diet high in omega-3 PUFA or omega-3 PUFA supplements during pregnancy could reduce the use of labour inducing drugs or the need for caesarean section which in turn would significantly reduce the government's healthcare costs.

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